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THE PHENOTYPIC MODIFICATION OF HEREDITARY POLYDACTYLISM OF FOWL BY SELECTION AND BY INSULIN*

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IT IS known from earlier investigations that hereditary polydactylism of fowl has a variable phenotype. The present report deals with two separate attempts to modify experimentally the expression of this mutation. In the first part we shall relate the results of matings which were made in order to test the effect of selection on several distinct phenotypes of polydactylism. In the second part a description will be given of the effects which the injection of insulin, at various stages of development, has on the expression of this mutation.

SELECTION EXPERIMENTS

The polydactylous or five-toed condition, which is typical of such breeds as Houdans, Dorkings and Silkies, is transmitted as a dominant trait (BATESON 1902; BATESON and PUNNETT 1905; HURST 1905; DUNN and JULL 1927; PUNNETT and PEASE 1929; WARREN 1944). All authors agree that the genetic transmission of polydactylism shows irregularities which have not been explained satisfactorily. There is no reason to believe that these breeds differ genetically in regard to polydactylism; in fact, what little is known about the history of Houdan and Dorking fowl suggests common ancestry for the polydactylous trait.

Within the five-toed breeds inheritance and morphological expression of the polydactylous condition show a high degree of regularity, although occasional individuals have an aberrant type of toe development or lack the extra toes altogether. But whenever representatives of the polydactylous breeds are crossed with normal-toed birds the resulting F_2 and further crossbred progenies tend to give irregular segregation ratios and show a great array of morphological variants of the polydactylous condition. Those variants include polyphalangism, that is the presence of a lengthened in place of a duplicated first toe, and heterodactylism, that is the presence of an extra toe on one but not on the other foot or a greater amount of duplication on one side as compared with the other. Throughout this report the term heterodactylism is limited to the association of one polydactylous or polyphalangeal and one normal foot. In order to avoid clumsy or misleading terminology we shall (if linguistically improperly) designate as "sinistral" heterodactylism presence of a hyperdactylous condition on the left, but not on the right, foot and as "dextral" heterodactylism the reverse condition.

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It was the purpose of our selection experiments to determine whether or not some of the special forms of expression of polydactylism are determined or influenced by specific genetic factors. The foundation material for our matings was obtained from DR. D. C. WARREN. It was a crossbred stock in which the polydactylous condition had originated from Silkie Bantam and Houdan fowl. Crosses of these chickens to White Leghorns furnished the material for the matings which will be discussed below. In addition, we have data from Dorking fowl matings.

The variation in expression of polydactylism in crossbred matings is far too great to allow of any but a somewhat arbitrary classification. We have used the following rules. All cases in which both feet showed more than four toes were called bilaterally hyperdactylous. Also included in this group were those birds in which one foot was polydactylous and the other polyphalangeal. Among the bilaterally polyphalangeal progenies we counted, in addition to uncomplicated cases, those in which one or both of the lengthened first toes had either slight lateral outgrowths or terminal duplications which did not involve more than the distal-most phalanx. Finally, the heterodactylous groups include all animals which had unilateral polydactylism, whether it was of the polyphalangeal type or a duplication *sensu stricto*. Our data consist of descriptive records from hatched chicks and embryos which had died during the last four days of incubation.

A few general observations are in order before we turn our attention to the results of selection for specific types of polydactylism. In the first place, our data give some information about the occurrence of segregation ratios with an excess of non-polydactylous chicks. In matings in which all parents were homozygous for polydactylism (matings 7-1942, 5-1943, 5-1944, 27-1945 in table 1; 4-1941, 4-1942, 4-1943 and 4-1944 in table 2) 12 non-polydactylous chicks or 0.5 percent were found among a total of 2440 chicks. Among 610 Dorking chicks (table 9, controls) the incidence of non-polydactylous progeny amounted to 1.3 percent. Matings in which all parents were heterozygous for polydactylism and had a *bilaterally polydactylous* or *bilaterally polyphalangeal* phenotype (matings 33-1939, 31-1941, 27-1942, 27-1943, 27-1944, 22-1941 in table 1 and 19-1939 in table 2) gave a segregation of 2382 polydactylous and 846 non-polydactylous chicks with an expectation of 2421 and 807, respectively. The deviation from a 3:1 expectation is not significant ($\chi^2 = 2.513$). In matings of heterozygous parents which were *heterodactylous* (data of tables 3 and 4), on the other hand, the progenies were composed of 2394 polydactylous and 1633 non-polydactylous chicks, where the expectation for a 3:1 ratio was 3020 and 1007, respectively. The deficiency in polydactylous offspring is statistically highly significant ($\chi^2 = 518.9$). This shows that heterozygous parents with a less pronounced phenotypic expression of polydactylism have a higher percentage of non-polydactylous progeny (normal overlaps) than those with a more extreme phenotype. It follows that the two groups must differ genetically.

Our data also demonstrate that heterodactylism occurs only as a rare exception in animals which are homozygous for polydactylism. The four matings of homozygous and bilaterally polydactylous parents listed in table 1 (matings

7-1942, 5-1943, 5-1944 and 27-1945) produced no heterodactylous chicks among 723 offspring. Another mating, in which all parents had six toes on each foot (table 8, controls), produced no heterodactylous chicks in a progeny of 273. Matings of homozygous, bilaterally polyphalangeal birds (4-1941, 4-1942, 4-1943 and 4-1944 in table 2) yielded 0.16 percent heterodactylism among a total of 1717 chicks, and a pen of Dorking fowl (table 9, controls) had 3.1 percent heterodactylous offspring in a progeny of 610 chicks. In contrast to these results, we found that those two matings of heterozygous and bilaterally polydactylous parents (33-1939 and 22-1941), in which little selection for any one particular type of expression of polydactylism had taken place, gave 13.0 percent heterodactylism among 794 hyperdactylous chicks. It can be concluded, as was suggested previously by BOND (1920), that as a rule heterodactylism is a heterozygous form of expression of polydactylism, and that, similar to the non-polydactylous overlaps, it occurs only rarely in homozygous polydactylous chicks.

PUNNETT and PEASE (1929) observed in their (rather small) material that matings which produced no chicks with dextral heterodactylism had fewer non-polydactylous offspring than occurred among progenies containing individuals with dextral heterodactylism. Such an association, if real, would be of considerable interest. We, therefore, tabulated our data from matings of heterozygous parents according to the incidence of dextral heterodactylism. The following frequencies of non-polydactylous progeny were found in the four groups.

Incidence of dextral heterodactylism in percent of all hyperdactylous	Total number of chicks	Non-polydactylous %
0	791	41.8±1.75
0.1–2.0	3932	32.6±0.74
2.1–4.0	912	31.7±1.55
>4.0	1688	44.1±1.25

Obviously there i no consistent trend. The difference in incidence of non-polydactylous chicks between progenies without cases of dextral heterodactylism and those in which more than four percent of the hyperdactylous chicks were dextrally heterodactylous was not significant. We conclude, therefore, that such differences as exist are spurious in nature.

The first of our selection lines was established for the purpose of breeding for a high incidence of the bilaterally five-toed condition. The birds for further matings were chosen each year from the progenies of those parents which had given the highest incidence of this condition. The relative frequency of bilaterally polydactylous chicks rose from an initial 82.5 to about 94 percent of all hyperdactylous offspring. These percentages include all forms of bilateral hyperdactylism, except bilateral polyphalangism. The effect of selection is not impressive, though undoubtedly real. At the end of this series of experiments all birds still were heterozygous for the polydactylism gene. This is explained by the subsequent observation that in our material homozygosity for poly-

dactylism was associated with the presence of more than five toes on each foot.

In 1941 a new line was established in which we selected for the occurrence of six toes on each foot (lower part of table 1). It can be seen that the incidence of animals with more than five toes on each foot rapidly rose from an initial 6.6 percent to 75.6 percent in the last generation. All animals of the last four generations were homozygous for polydactylism; bilateral polyphalangism was

TABLE 1

Selection for bilateral expression of polydactylism. The upper part of the table shows results of selection for five toes on each foot; the lower part gives the data of selection for the presence of more than five toes on each foot. Figures in parenthesis give incidence of chicks with more than five toes in percent of all polydactylous.

MATING AND YEAR	TOTAL PROGENY	TOTAL POLYDACTYLOUS	IN PERCENT OF ALL POLYDACTYLOUS			
			BILATERAL POLYDACTYLISM	BILATERAL POLYPHALANGISM	SINISTRAL HETERODACTYLISM	DEXTRAL HETERODACTYLISM
33-1939	685	492	82.5	7.3	8.3	1.8
28-1940	133	120	82.5	1.7	13.3	2.5
31-1941	614	473	94.3	2.1	3.2	0.4
27-1942	360	275	93.8	5.8	0.4	0
27-1943	314	259	88.8	10.4	0.8	0
27-1944	440	322	94.1	3.4	2.2	0.3
22-1941	416	302	82.5 (6.6)	0	14.2	3.3
7-1942	155	154	99.4 (31.2)	0.6	0	0
5-1943	215	214	100 (55.6)	0	0	0
5-1944	218	218	96.3 (72.0)	3.7	0	0
27-1945	135	135	94.8 (75.6)	5.2	0	0

rare, but the form of expression of bilateral hyperdactylism remained very variable.

The results of selection for bilateral polyphalangism are shown in table 2. Beginning with 1940 all animals in our matings were homozygous for hyperdactylism. The incidence of bilateral polyphalangism rose from an initial 68.3 percent (of all hyperdactylous) to 88.6 percent at the end of the experiment. This increase was accomplished by the virtual disappearance of heterodactylism and by a gradual decline in the frequency of bilaterally polydactylous animals. It must be added, however, that entirely uncomplicated bilateral polyphalangism was, in our material, more commonly found in matings of

heterozygous than of homozygous animals. As the parents became homozygous for hyperdactylism one or both first toes, in a high percentage of the polyphalangous progeny, showed duplication of the toe nail or of the first phalanx or small lateral outgrowths in varying positions. The lengthening of the first toe continued to exist, but was at the same time complicated by a trend toward duplication. Polyphalangism without duplication tends to be limited to heterozygotes. This accounts for our failure to establish a line which breeds true for uncomplicated bilateral polyphalangism. In an outcross of a homozygous, bilaterally polyphalangeal male to Leghorn females (table 6, mating 19-1940) the incidence of bilateral polyphalangism amounted to only 5.1 percent compared with 69.2 percent when the same cock was bred to homozygous, bilaterally polyphalangeal females.

TABLE 2

Results of selection for bilateral expression of polyphalangism.

MATING AND YEAR	TOTAL PROGENY	TOTAL POLYDACTYLOUS	IN PERCENT OF ALL POLYDACTYLOUS			
			BILATERAL POLYDACTYLISM	BILATERAL POLYPHALANGISM	SINISTRAL HETERODACTYLISM	DEXTRAL HETERODACTYLISM
19-1939	399	259	25.5	68.3	5.0	1.2
19-1940	294	279	28.7	69.2	1.4	0.7
4-1941	496	488	20.9	77.0	1.8	0.2
4-1942	394	394	31.7	66.8	1.5	0
4-1943	494	492	11.8	87.0	0.8	0.4
4-1944	333	333	9.6	88.6	1.2	0.6

WARREN (1944) concluded from his data that polyphalangism is "a factor which behaves as an autosomal dominant with some unknown factors causing variability in dominance." Our observations cannot be reconciled with this interpretation. It seems likely, both from Warren's and our own results, that the modifying situation which produces the polyphalangous type of hyperdactylism has a relatively simple genetic basis, but the mode of transmission must differ considerably between stocks.

The results of selection for sinistral heterodactylism are reproduced in table 3. The matings consisted throughout of birds which were heterozygous for polydactylism since, as was stated previously, heterodactylism is as a rule a heterozygous expression of the mutation. In the course of seven generations of selection the incidence of sinistral heterodactylism rose from 12.6 percent to 33.1 percent of the hyperdactylous progenies. The difference equals 20.5 ± 4.06 percent. This highly significant increase in sinistral heterodactylism occurred entirely or almost entirely at the expense of bilateral polyphalangism which disappeared nearly completely from the stock. The results seem to show clearly that the modifying agencies which are responsible for bilateral polyphalangism and for sinistral heterodactylism are incompatible. The incidence of bilateral polydactylism was not affected, nor did selection have any notice-

able effect on the frequency with which dextral heterodactylism occurred. It should be noted that in three matings of heterodactylous and polyphalangeal males with sinistral expression of the hyperdactylous condition to Leghorn females (matings 31-1940, 16-1942 and 32-1938 in table 6) the incidence of sinistral heterodactylism remained relatively high in the resulting progenies (29.0, 13.3 and 28.9 percent, respectively). This may indicate some degree of dominance of the modifying factors which produce heterodactylism, but is

TABLE 3

Results of selection for sinistral heterodactylism.

MATING AND YEAR	TOTAL PROGENY	TOTAL POLYDACTYLOUS	IN PERCENT OF ALL POLYDACTYLOUS			
			BILATERAL POLYDACTYLISM	BILATERAL POLYPHALANGISM	SINISTRAL HETERODACTYLISM	DEXTRAL HETERODACTYLISM
27-1938	185	119	64.7	21.8	12.6	0.8
31-1939	683	436	66.1	14.7	17.9	1.4
31-1940	117	63	69.8	4.8	25.4	0
15-1941	203	121	68.6	2.5	25.6	3.3
16-1942	293	200	72.5	3.0	21.5	3.0
3-1943	428	250	72.0	1.2	25.6	1.2
3-1944	498	299	65.6	0.7	33.1	0.7

TABLE 4

Results of selection for dextral heterodactylism.

MATING AND YEAR	TOTAL PROGENY	TOTAL POLYDACTYLOUS	IN PERCENT OF ALL POLYDACTYLOUS			
			BILATERAL POLYDACTYLISM	BILATERAL POLYPHALANGISM	SINISTRAL HETERODACTYLISM	DEXTRAL HETERODACTYLISM
20-1941	243	129	63.6	3.9	24.0	8.5
8-1942	334	122	69.7	1.6	23.8	4.9
24-1943	265	134	68.7	5.2	18.7	7.5
24-1944	364	243	72.0	2.5	16.0	9.5
1-1945	149	100	66.0	2.0	17.0	15.0
1-1946	265	178	56.7	12.9	19.1	11.2

more likely a result of the presence of these modifying factors in our (outbred) polydactylous as well as the Leghorn stock.

The data from our line selected for dextral heterodactylism are presented in table 4. It can be seen that selection did not produce a pronounced or consistent change in the incidence of dextral heterodactylism, although there appears to be some trend toward a higher frequency of this type of hyperdactylism. A comparison of the first three and the last three generations of selection gives a difference in dextral heterodactylism of 4.01 ± 1.90 percent in favor of the later generations. This is on the border line of statistical signifi-

cance. A comparison of the data in table 4 with those of tables 3 and 6 shows, however, that every one of the matings of heterozygous and dextrally heterodactylous parents produced a higher incidence of dextrally heterodactylous offspring than any of the matings of heterozygous parents with hyperdactylism other than dextral heterodactylism. If the results for all sinistrally heterodacty-

TABLE 5

Comparison of over-all incidence of dextral heterodactylism, in percent of all polydactylous, in the lines selected for sinistral and dextral heterodactylism, respectively.

SELECTION FOR	TOTAL POLY-DACTYLOUS	INCIDENCE OF DEXTRAL HETERO-DACTYLISM %	DIFFERENCE IN %
Sinistral heterodactylism	1488	1.5±0.31	8.1±1.01
Dextral heterodactylism	938	9.6±0.96	

TABLE 6

Results of outcrosses.

TYPE OF CROSS	MATING AND YEAR	TOTAL PROGENY	TOTAL POLYDACTY-LOUS	IN PERCENT OF ALL POLYDACTYLUS			
				BILATERAL POLYDACTY-LISM	BILATERAL POLY-PHALANGISM	SINISTRAL HETERO-DACTYLISM	DEXTRAL HETERO-DACTYLISM
Leghorn×bilaterally polydactylous	28-1940	106	90	82.2	0	14.4	3.3
Leghorn×bilaterally polyphalangeal	19-1940	161	157	84.7	5.1	5.7	4.5
Leghorn×sinistrally heterodactylous	31-1940	194	69	62.3	1.4	29.0	1.2
	16-1942	221	105	86.7	0	13.3	0
Leghorn×sinistrally polyphalangeal	32-1938	532	194	53.1	16.5	28.9	1.5
Leghorn×dextrally heterodactylous	11-1940	352	169	81.1	1.2	15.4	2.4
	20-1941	488	232	83.6	5.6	8.2	2.6

lous (table 3) and all dextrally heterodactylous (table 4) parents are compared with reference to the frequency of dextral heterodactylism among the progenies a difference of 8.1±1.01 percent is found in favor of the matings of dextrally heterodactylous parents (table 5). This difference is highly significant. It cannot be doubted, therefore, that genetic factors influence the occurrence of dextral heterodactylism.

THE EFFECT OF INSULIN

Our experiments with insulin were designed to study the effect of this hormone on the expression of polydactylism. Iletin (Insulin, Lilly) was injected into eggs at 24-hour intervals between 24 and 144 hours of incubation. The details

of technique were the same as described earlier (LANDAUER 1945). Eggs from three different matings were used as follows: (a), Leghorn pullets mated to cocks of Silkie-Houdan-Leghorn crossbred origin which were homozygous for polydactylism and had six toes on each foot; (b), *inter se* matings of homozygous polydactylous fowl of Silkie-Houdan-Leghorn crossbred origin, all of which were bilaterally six-toed; (c), *inter se* matings of Silver Gray Dorking fowl. For each of the developmental test stages and the three sources of origin we had groups of eggs injected with two and five units of insulin and untreated

TABLE 7

Insulin experiments with eggs from matings of Leghorn pullets and homozygous polydactylous, bilaterally six-toed cocks of Silkie-Houdan-Leghorn crossbred origin.

TREATMENT	TIME OF INJECTION	N	BILATERAL POLYDACTYLISM	NOT BILATERALLY POLYDACTYLOUS	HETERODACTYLISM	BILATERAL POLYPHALANGISM	UNI- OR BILATERAL ECTRODACTYLISM	NORMAL
			%	%	%	%	%	%
2 units insulin	24	73	68.5	31.5	8.2	19.2	1.4	2.7
	48	78	79.5	20.5	2.6	15.4	1.3	1.3
	72	100	46.0	54.0	8.0	40.0	0	6.0
	96	95	43.2	56.8	8.4	34.7	0	13.7
	120	74	81.1	18.9	2.7	16.2	0	0
	144	106	91.5	8.5	0.9	7.5	0	0
5 units insulin	24	67	77.6	22.4	4.5	16.4	0	1.5
	48	102	66.7	33.3	2.9	28.4	1.0	1.0
	72	102	52.0	48.0	2.9	35.3	1.0	8.8
	96	101	35.6	64.4	9.9	37.6	0	16.8
	120	77	81.8	18.2	0	15.6	0	2.6
	144	113	85.0	15.0	0.9	14.2	0	0
Untreated controls for stated periods of injection	24	130	83.1	16.9	0.8	16.2	0	0
	48	162	87.0	13.0	0.6	9.3	0	3.1
	72	119	85.7	14.3	0.8	13.4	0	0
	96	114	90.4	9.6	0	9.6	0	0
	120	78	84.6	15.4	0	15.4	0	0
	144	110	82.7	17.3	1.8	15.5	0	0

controls. The results of these experiments are reproduced in tables 7 to 9.

In the absence of experimental interference all three types of matings gave progenies with a high incidence of bilateral polydactylism. Among the F_1 offspring of Leghorn and homozygous polydactylous fowl 83.1 to 90.4 percent were bilaterally polydactylous (mean of aberrant types 14.3 ± 1.31 percent); 83.3 to 95.3 percent bilaterally polydactylous progeny were found among the offspring of *inter se* matings of homozygous polydactylous chickens of crossbred origin (mean of aberrant types 13.9 ± 2.11 percent), and for the Dorking progenies the corresponding figures were 93.3 to 100 percent (mean of aberrant types 4.6 ± 0.84 percent).

The over-all effect of insulin is shown by the totals of all chicks which were not bilaterally polydactylous. It can be seen that with all three stocks and with both dosages of insulin the maximum deviations from the normal incidence of bilateral polydactylism occurred at 72 and 96 hours. At the 120 and

144 hour test periods insulin did no longer change the expression of polydactylism. It is evident, on the other hand, that even at the earliest developmental stages which were exposed to insulin, 24 and 48 hours, the treatment produced an appreciable effect.

It is true for all three stocks that the two concentrations of insulin (2 and 5 units) did not result in consistently different results. This holds for the totals

TABLE 8

Insulin experiments with eggs from inter se matings of homozygous polydactylous, bilaterally six-toed fowl of Silkie-Houdan-Leghorn crossbred origin.

TREATMENT	TIME OF INJECTION	N	BILATERAL POLYDACTYLISM %	NOT BILATERALLY POLYDACTYLOUS %	HETERODACTYLISM %	BILATERAL POLYPHALANGISM %	UNI- OR BILATERAL ECTRODACTYLISM %	NORMAL %
2 units insulin	24	22	59.1	40.9	0	40.9	0	0
	48	18	66.7	33.3	0	27.8	5.6	0
	72	21	38.1	61.9	0	61.9	0	0
	96	23	39.1	60.9	4.3	52.2	0	4.3
	120	17	88.2	11.8	0	11.8	0	0
	144	43	93.0	7.0	0	7.0	0	0
5 units insulin	24	13	61.5	38.5	0	38.5	0	0
	48	16	56.3	43.8	0	43.8	0	0
	72	19	26.3	73.7	0	63.2	0	10.5
	96	16	37.5	62.5	0	43.8	0	18.8
	120	17	88.2	11.8	0	11.8	0	0
	144	37	94.6	5.4	2.7	2.7	0	0
Untreated controls for stated periods of injection	24	38	84.2	15.8	0	15.8	0	0
	48	47	85.1	14.9	0	14.9	0	0
	72	60	85.0	15.0	0	15.0	0	0
	96	43	95.3	4.7	0	4.7	0	0
	120	30	83.3	16.7	0	16.7	0	0
	144	55	83.6	16.4	0	14.5	0	1.8

of all aberrations from bilateral polydactylism as well as for the various individual types of expression. Furthermore, there is no consistent difference in the effects obtained at 72 and 96 hours. For statistical analysis it, therefore, seemed justified to combine within each of the three stocks all data obtained for two and five units of insulin at both 72 and 96 hours of incubation.

In regard to the extent of the insulin effect at the period of maximum action (72 and 96 hours) the following differences were found between the treated (2 and 5 units insulin) and control groups in percent of progeny which was not bilaterally polydactylous:

F_1 (Leghorn×outbred homozygous polydactylous)	43.8±3.27 percent
Outbred homozygous polydactylous *inter se*	53.9±6.14 percent
Dorking	20.9±3.13 percent

It is evident that insulin produced in all three stocks a very striking and highly significant departure from the normal incidence of bilateral polydactylism. It can be seen, furthermore, that these insulin-produced phenotypic shifts were similar in extent in the heterozygous and homozygous progenies of

Silkie-Houdan outbred ancestry, but that in the Dorking stock the response was much less extreme.

We have already pointed out that even in the earliest stages which we tested insulin had some effect on the expression of polydactylism. The combined data for the 24 and 48 hour stages and the two dosages gave the following differ-

TABLE 9

Insulin experiments with eggs from Dorking fowl.

TREATMENT	TIME OF INJECTION	N	BILATERAL POLYDACTYLISM %	NOT BILATERALLY POLYDACTYLOUS %	HETERODACTYLISM %	BILATERAL POLYPHALANGISM %	UNI- OR BILATERAL ECTRODACTYLISM %	NORMAL %
2 units insulin	24	48	85.4	14.6	4.2	0	0	10.4
	48	69	89.9	10.1	1.4	0	2.9	5.8
	72	80	78.8	21.3	5.0	1.3	0	15.0
	96	65	70.8	29.2	12.3	1.5	0	15.4
	120	58	94.8	5.2	0	1.7	0	3.4
	144	55	96.4	3.6	3.6	0	0	0
5 units insulin	24	56	82.1	17.9	8.9	0	0	8.9
	48	72	88.9	11.1	5.6	0	0	5.6
	72	69	76.8	23.2	13.0	0	0	10.1
	96	61	67.2	32.8	8.2	0	0	24.6
	120	45	100	0	0	0	0	0
	144	55	92.7	7.3	3.6	1.8	0	1.8
Untreated controls for stated periods of injection	24	90	96.7	3.3	2.2	0	0	1.1
	48	136	93.4	6.6	5.9	0	0	0.7
	72	117	94.9	5.1	3.4	0.9	0	0.9
	96	104	93.3	6.7	2.9	0	0	3.8
	120	84	96.4	3.6	2.4	0	0	1.2
	144	79	100	0	0	0	0	0

ences between treated and control groups in incidence of chicks which were not bilaterally polydactylous:

F_1 (Leghorn×outbred homozygous polydactylous)	12.8±3.26 percent
Outbred homozygous polydactylous *inter se*	23.8±6.99 percent
Dorking	5.3±1.49 percent

The differences are significant for all three types of matings. In these early stages, as in the later ones which were discussed above, the Dorkings gave a much less pronounced response than the stocks of outbred origin.

At 120 and 144 hours of development the insulin effect has disappeared. A comparison within the treated groups (2 and 5 units insulin) of the values for the maximum response periods (72 and 96 hours) and the late stages (120 and 144 hours) gave the following differences in frequency of non-bilaterally polydactylous offspring:

F_1 (Leghorn×outbred homozygous polydactylous)	41.2±3.08 percent
Outbred homozygous polydactylous *inter se*	56.7±5.90 percent
Dorking	22.0±3.01 percent

These values are very similar to those given above for the differences between

treated and control groups at the period of maximum response, indicating the disappearance of the insulin effect in the late stages.

An examination of the data in tables 7 to 9 shows interesting differences between the three stocks in regard to the specific types of deviations from bilateral polydactylism which are produced by insulin. Among the untreated progenies from the outbred stock, whether hetero- or homozygous, bilateral polyphalangism is the most common aberration, and bilateral polyphalangism is also by far the most frequent aberrant phenotype in the insulin-treated material. In the Dorking stock, on the other hand, bilateral polyphalangism is very rare. It remains similarly rare after the injection of insulin. Heterodactylism was absent from the control data of outbred homozygous polydactylous origin and very rare in the insulin-treated material. Heterodactylism did occur among the control chicks of the outbred heterozygous polydactylous and the Dorking stocks and insulin significantly increased its incidence. Normal, that is non-polydactylous, chicks were more common among Dorking progenies than in the two other stocks and the same was true for the insulin data. It should be observed, however, that heterodactylism was found more

TABLE 10

Differences between insulin-treated (72 and 96 hours; 2 and 5 units) and control groups in incidence of various phenotypic expressions of polydactylism.

PHENOTYPE	F_1 (LEGHORN×OUTBRED HOMOZYGOUS POLYDACTYLOUS)	OUTBRED HOMOZYGOUS POLYDACTYLOUS *inter se*	DORKING
Heterodactylism	6.9±1.37	1.3±1.31	6.8±2.14
Bilateral polyphalangism	25.3±3.20	45.0±6.33	0.7±0.56
Normal	11.1±1.59	7.6±2.98	13.3±2.52

commonly than the normal condition among the controls of the outbred heterozygous polydactylous and Dorking chicks, but that in the insulin-treated material these positions were reversed, that is, the incidence of normal chicks in both instances was more exaggerated than that of heterodactylous ones. All these conclusions are illustrated by the data of table 10.

Attention must finally be called to the fact that in a number of instances ectrodactylism was observed in the insulin-treated material. All these cases occurred after the injection of insulin between 24 and 72 hours of development. For the three stocks and the two concentrations of insulin we had 1025 chicks from eggs which had been injected between 24 and 72 hours with an incidence of 0.68±0.26 percent of ectrodactylism. No cases of ectrodactylism were found among 899 chicks from control eggs incubated during the same periods. It is important to observe, however, that ectrodactylism is more likely to occur in polydactylous than in other stocks. Between 1938 and 1946 we found 8 cases of ectrodactylism (0.069±0.024 percent) among 11,654 chicks from matings of polydactylous fowl. No ectrodactylous chicks occurred among 1760 chicks obtained during the same period from non-polydactylous parents which

had been extracted from matings of heterozygous polydactylous fowls, nor were any ectrodactylous chicks met with among large numbers of Leghorn chicks hatched in the course of the same years.

DISCUSSION

The high degree of variability in the expression of polydactylism of fowl has been noted by all observers who have worked with this trait. All attempts to fit the phenotypes into a small number of distinct morphological groups do violence to the actual situation. A series of imperceptible steps links the four-toed with the six-toed condition and the five-toed phenotype with hyperphalangism. The same may even be said in regard to heterodactylism and bilateral hyperdactylism (plate I). Any attempt to study specific variants of hyperdactylism must, therefore, deal with trends in one or another direction of phenotypic expression and must delimit the end-points of these series from intermediate forms.

The data of earlier investigators have already given some indication that hereditary factors influence the specific expression of polydactylism. This is particularly true for the observations of WARREN (1944) on polyphalangism and several modes of expression of bilateral polydactylism. The data of the present report lead to the following conclusions:

1. Heterodactylism is generally a heterozygous expression of polydactylism, but occurs among homozygotes as a rare variant.

2. The incidence of sinistral heterodactylism can be raised by selection.

3. Dextral heterodactylism, which in the absence of selection is much less common than the opposite form of asymmetry, shows also less response to selection. However, *inter se* matings of parents with dextral heterodactylism produce significantly more progeny with heterodactylism of this type than any other kind of mating.

4. Bilateral polyphalangism responds readily to selection. Uncomplicated bilateral polyphalangism occurs chiefly, perhaps exclusively, among heterozygous animals. In homozygous polyphalangeal chicks the lengthened first toe generally, if not always, shows some indication of duplication.

5. The incidence of bilaterally five-toed and bilaterally six-toed chicks can readily be increased by selection. The presence of five toes on both feet was a heterozygous expression in our material of outbred origin, but was the rule among homozygous pure-bred Dorking fowl. Homozygous birds of our outbred stock had, almost without exception, more than five toes on one or both feet.

6. Our data, as those of earlier observers, show that non-polydactylous chicks are, on rare occasions, found among the progenies of homozygous polydactylous fowl. Heterozygous polydactylous parents with a low degree of ex-

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PLATE I.—Hyperdactylous feet, selected to illustrate the transgressive nature of variation of this trait. Top row: transition from four-toed to five-toed condition; middle row: stages leading from the five-toed to the six-toed phenotype; bottom row: steps connecting hyperphalangism with the five-toed expression of polydactylism.

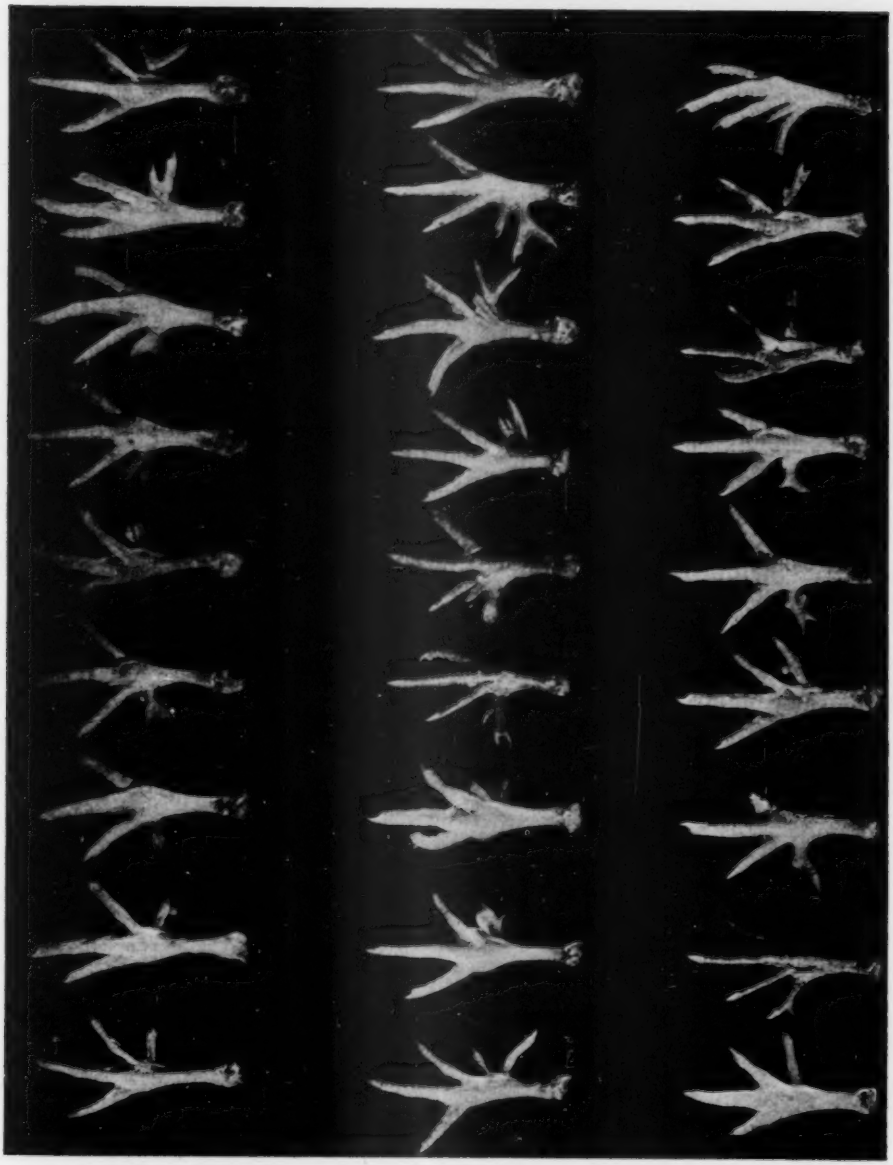

PLATE I. See opposite page for description.

PLATE II. See opposite page for description.

pression of polydactylism (heterodactylism) produce significantly more non-polydactylous offspring than heterozygous parents with a higher degree of gene expression (bilateral polydactylism or bilateral polyphalangism).

These observations demonstrate beyond doubt that all the different forms of expression of polydactylism are influenced by hereditary factors. It is equally clear that none of the modifications are brought about by simple genetic changes. The transgressive nature of the variations points to the conclusion that the various forms of expression of polydactylism arise on the basis of developmental processes which can easily be disturbed and pushed into one of several directions. It seems likely, furthermore, that these processes are of a quantitative nature.

One of the most interesting aspects of polydactylism is the occurrence of unilateral gene expression, that is heterodactylism. All observers agree that sinistral heterodactylism is much more common than the opposite form (BOND 1920, 1925–1926; PUNNETT and PEASE 1929; VAINIKAINEN 1935; WARREN 1944; data of the present report), although it was shown by PUNNETT and PEASE that this trend may be reversed in exceptional matings. It was already observed by BOND and is confirmed by our own observations that the same kind of asymmetry is found in cases of unequal expression of bilateral hyperdactylism, that is an excess on the left side is much more common than the opposite situation. Furthermore, this generalization also holds for expression of polydactylism in the upper extremities. From our Silkie-Houdan-outbred stock a line has been produced by selection in which supernumerary digits are prominently developed in the wings (unpublished). Such well-developed supernumerary fingers occur only in birds which are homozygous for polydactylism. Sinistral heterodactylism of the wings is much more common than the dextral form of expression.

It is of considerable interest that conditions of asymmetry similar to those in fowl have been found in guinea pigs. It is always true that among the cases of asymmetrical expression extra toes occur more often on the left than on the right side (CASTLE 1906; PICTET 1932; KRÖNING and ENGELMANN 1934; WRIGHT 1934). Further, similar to the situation in poultry, KRÖNING and ENGELMANN observed that among cases of asymmetrical bilateral polydactylism in guinea pigs more pronounced sinistral development occurred with higher frequency than the opposite type. They also found that bilaterally

PLATE II. See opposite page for description.

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PLATE II.—Examples of ectrodactylism from insulin-treated embryos. 1. First toe hyperphalangous on both feet; second toe double nail, third toe of left foot rudimentary, fourth toe of right foot absent, small toe rudiment with nail attached to tip of third toe, syndactyly of second and third toe. 2. Both feet show lack of first and partial duplication of second toe. 3. Left foot partial duplication, right foot incomplete triplication of second toe; first toe missing on both feet (on right foot the lateral component of the fused toe mass may represent the first toe). 4. Left foot five-toed; on right foot first toe missing, second toe partially duplicated.

polydactylous animals of their family with the highest incidence of sinistral heterodactylism commonly showed more digit development on the left side. Attempts by CASTLE to increase by selection the asymmetry of the two sides of the body with reference to the extra toe were unsuccessful.

BOND (1932), speculating on the causes of heterodactylism, suggested the occurrence of unequal distribution of gene material during early developmental stages. He said: "the fact that an old established racial character is, for the most part, distributed *equally* to the two halves of the organism, while a newly introduced, (such as an extra toe), is often distributed *unequally*, suggests the idea that the new factor, or gene, fuses with difficulty with the older established complex. In other words that the old and new factors tend to segregate during the process of cell division which underlies the bilateral development of the embryo. The result being that asymmetrical distribution of the extra toe character occurs on the two sides of the body." And again: "It looks indeed as if a condition of unstable equilibrium has been set up in the genetic machinery which controls embryological development, by the introduction of a foreign element or character into an old established racial genetic complex, and that this disturbance of equilibrium is in some way associated with the abnormal distribution of the character in question, to the two sides of the body."

The occurrence of such a bilateral gene segregation is unlikely on general grounds. Breeding data show that the ovary (left side of body) is not involved in such a segregation. The preferential expression of polydactylism on the left side, among cases of heterodactylism, would remain unexplained. The fact that the left body side tends to show the higher degree of polydactylous development in bilateral, but unequally expressed, hyperdactylism precludes the assumption of an unequal distribution of the main gene for polydactylism, and it would seem unreasonable to invoke different explanations for unilateral and for asymmetrical bilateral polydactylism. Finally, the fact that heterodactylism of the fingers is common in birds which are homozygous polydactylous would seem to dispose of any explanation based on unequal gene distribution to the two body sides.

The only other attempt to provide an explanation for the developmental mechanism of heterodactylism has been made by DAHLBERG (1929, 1943 a, b). His hypothesis is based on the assumption that symmetrically distributed genes may produce a reversibly asymmetrical organization of the soma. DAHLBERG refers to such conditions as "genotypical asymmetries" and believes that their explanation is likely to be as follows:

1. "That a particular gene may lead to unequal distribution of some cytoplasmic inclusion at a determinative cell division which occurs in the plane of lateral symmetry."

2. "That it is a matter of chance whether excess or deficiency of the cytoplasmic constituent is concentrated on one side or other of the partition formed at the determinative cell division."

3. "That the exhibition or suppression of what would otherwise be a sym-

metrical character is limited by this excess or deficiency in the descendants of cells from the determinative division."

In applying these ideas to heterodactylism of fowl DAHLBERG starts with the entirely unjustified assumption that "among descendants of heterozygotes which have the fifth toe on the left side, asymmetrical offspring with the fifth toe on the right side appear to turn up as often as the parental type," and that "orientation of the character is not fixed by the process of transmission." As has been pointed out earlier, all observers are agreed that (in the absence of specific selection) the incidence of sinistral heterodactylism is far in excess of cases of dextral heterodactylism. Our own observations furnish definite proof for the existence of genetic factors which favor the appearance of one or the other type of heterodactylism. One would have to make very complex and improbable assumptions to fit DAHLBERG'S hypothesis to the following established facts: (a) the left-right incidence of heterodactylism is not a chance event, but is influenced by the residual heredity of the embryo; (b) heterodactylism occurs, if rarely, in homozygous individuals and the incidence in homozygotes can be greatly increased by experimental manipulation (see below); (c) heterodactylism of the upper extremities may co-exist with symmetrical bilateral gene expression in the toes of individuals which are homozygous for polydactylism.

Two other possibilities of explanation, envisioned but rejected by DAHLBERG, viz., factors extrinsic to the embryo and chromosomal abnormalities (such as elimination or non disjunction of chromosomes), are equally in conflict with factual observations and do not need to be discussed here.

We believe that all variations in the expression of polydactylism of fowl can be understood on the basis of known differentials in the internal environment of the embryo. K. E. VON BAER (1828) first observed that during the third and fourth days of development (the period of origin of the limb buds) the whole right half of the body grows at a more rapid rate than the left side. This was confirmed by subsequent investigations and BRANDT (1913) called particular attention to the fact that the limb buds are included in this lateral asymmetry of growth. There is, however, reason to believe that the situation is much more complex than these observations would suggest. OLSEN and BYERLY (1935) found, by counting mitoses on the left and right side of the neural tube, that in embryos of eleven to sixteen somites the left side showed a higher rate of growth, but that the reverse, that is higher mitotic activity on the right side, obtained in somewhat later stages. This change from left to right in preferential growth intensity coincides with turning of the embryo on to its left side. It cannot be doubted that such differential growth rates, whether they are lateral or regional, are an important expression of morphogenetic events. They deserve intense quantitative study.

An expression of these lateral differences in growth intensity was found by RAWLES (1936) in a comparative study of the fate (in chorioallantoic transplants) of pieces from the head-process stage of chick blastoderms, that is, at a time when growth intensity is higher on the left side. She observed that in

comparison with grafts from the median region of the embryo those of lateral origin had a reduced developmental capacity (in respect to growth and differentiation), and that this reduction was consistently greater for pieces from the right side than for those from the left. It is evident that growth activity at the time of explantation played an important role in determining the fate of the explant. Similar observations have been made in amphibian embryos. For a general discussion of the bearing of early activity gradients on the origin of lateral asymmetry and inversion the reader is referred to HUXLEY and DEBEER (1934, chapter IV) and PERNKOPF (1937).

We suggest that these lateral asymmetries of embryonic growth, their reversal in the case of heterotaxia and their more detailed and as yet unknown local variations provide the complex mechanism by which the particular type of expression of polydactylism or its complete suppression is mediated. One would assume that such lateral or local growth differentials are influenced by multiple genetic factors and this is, in fact, supported by the results of our selection experiments. If the results of selection in one or another direction of the phenotypic manifestations of polydactylism are obtained by the accumulation of factors for certain types of specific growth patterns, one would assume the emergence of antagonistic relationships between some forms of expression. Such incompatibility was demonstrated for bilateral polyphalangism and sinistral heterodactylism.

We believe that an interpretation of this kind is strongly supported by the nature of those external agencies which have a modifying effect on polydactylism. STURKIE (1943) and WARREN (1944) observed that the expression of polydactylism can be suppressed or shifted to different phenotypes by exposing the developing embryos to low temperatures. This procedure was most effective during the third day of incubation and only slightly so by the fifth day. The data of STURKIE indicate that these results are brought about by developmental modifications of a quantitative nature. GABRIEL (1946) has shown that growth retardation of the whole limb anlage of homozygous polydactylous embryos, produced by treatment with colchicine, leads in a high percentage of cases to suppression of the supernumerary digits. Finally, there are the effects of insulin as related in this report. The period during which polydactylism can be modified by insulin, the time of its maximum action, and the phenotypic results are very similar to those obtained with low temperatures. It seems reasonable to assume that the three agencies, viz., low temperature, colchicine and insulin, produce their effects in the same way, viz., by depressing growth. It is likely that the growth retardations are differential and vary with conditions of local growth intensity. We do not know the causes which produce lateral asymmetries or local variations of growth rate in normal development, nor do we have any information about the specific biochemical events which may be affected by experimental conditions and thereby result in the assumed growth retardations. We do not imply that *every* agent which has a general retarding effect on growth will thereby produce the modifications to which we have referred. It is, in fact, quite possible that such retardations, in order

to be effective, must be mediated through specific channels which are concerned with particular aspects of growth.

Lateral asymmetries in the expression of hereditary traits, similar to those occurring in polydactylism of fowl, are found among widely different forms of higher vertebrates. We have already referred to the parallel observations for polydactylism of guinea pigs. Another instance of very similar nature is that of congenital absence of the palmaris longus muscle in man. The condition is hereditary (THOMPSON, MCBATTS and DANFORTH 1921). In cases of unilateral suppression the muscle is more often absent on the left than on the right side. Parents with unilateral absence may produce children with lack of the muscle on the same, the opposite, or both sides; parents with bilateral lack of the muscle may have children with unilateral defect. DANFORTH (1924) believes that "it is difficult to escape the conclusion that in some way the sides of the body, *per se*, exert an influence in determining whether or not a factor represented in the germ plasm will succeed in producing its full effect." With this inference we are in full accord.

Another case of interest is that of foot abnormalities found among the descendants of X-rayed mice. These abnormalities generally show a greater incidence on the left than on the right side of the body, but this asymmetry of expression could be overcome by selection (LITTLE 1931). We believe that the wide distribution of asymmetrical phenotypic manifestation of traits, with similar features of hereditary transmission, is a reflection of the equally wide distribution of lateral growth asymmetries in the embryonic development of the amniotes. Modifications of these growth rates may even account for the effect which nutrition and maternal age appear to have on phenotypic expression of polydactylism in guinea pigs (WRIGHT 1934; KRÖNING and ENGELMANN 1934).

The hereditary basis of embryonic growth differentials is presumably complex and if, in addition, these growth rates are subject to modification by external agencies, such as temperature or nutrition, it becomes more readily understandable that, as is the case in fowl, the factors which are responsible for asymmetrical phenotypes may be derived from non-polydactylous ancestors and transmitted by animals with bilateral expression of the gene.

It must not be concluded from the foregoing discussion that all abnormalities of the extremities which occur unilaterally as well as bilaterally should, among the unilateral variants, have a higher sinistral incidence. This is notably not true. An example of the opposite type which has a direct bearing on our discussion is that of "diplopodia," a recessive lethal mutation of fowl discovered by TAYLOR and GUNNS (1947). Embryos which are homozygous for the diplopod gene show partial doubling of the metatarsals and digits of the feet, the metacarpals and digits of the wings, shortening of the long bones and of the maxilla. There are generally six toes on each foot, but asymmetrical expression occurs. TAYLOR and GUNNS reported, and this is confirmed by observations of our own, that among diplopod embryos with asymmetrical toe duplication the greater abnormality is, in about five out of six embryos, found on the right

foot. Heterodactylism with unilateral duplication does not occur in this stock, but asymmetrical manifestation of the gene is of the opposite type from that found in ordinary polydactylism. It seems likely that such dissimilarities between mutant stocks are brought about by differences in the time at which the gene takes effect and by the relation of the determinative periods to the changing pattern of relative growth in various parts and regions of the embryo. Specifically, we suggest that in heterozygous polydactylous as well as homozygous diplopod embryos that side of the embryo is more resistant to the gene effect which at the determinative period shows more intense growth activity. It should be possible to test the correctness of these assumptions.

A few remarks should be made in regard to the modifying effect of insulin on polydactylism of fowl. The time of maximum effectiveness (72 to 96 hours of development) coincides with the time of origin and early organization of the limb buds. The fact that no dosage difference was produced by the injection of two and five units of insulin suggests that the maximum result is brought about by the lesser amount of the hormone. The results of our experiments, in comparison with the controls, indicate that insulin tends to push development into channels for which the embryos of a particular stock are prepared by their genotype.

It is of some interest to note that the insulin-induced phenotypic shift was similar in type and extent in the heterozygous and homogygous embryos of *outbred* origin and that the effect in these two stocks was much greater than that found for the *pure-bred* Dorkings. Differences of the same kind, though at much lower levels, exist between the *untreated* outbred and pure-bred stocks. While it is unquestionably true that certain forms of phenotypic expression of polydactylism or its complete suppression occur predominantly among heterozygotes, it is also evident that these effects are more likely to take place after the introduction of modifiers by outcrosses. The data of all investigators indicate that the frequency of occurrence and nature of these modifiers varies from stock to stock; they appear to be rare among Jungle fowl (BOND 1932; FISHER 1935).

It is obvious, of course, that in producing relatively true-breeding polydactylous races breeders have resorted to a great deal of selection. It has been suggested that the effect of this selection was to accumulate in the polydactylous stocks "dominance factors" which enhance the full expression of an originally semi-dominant mutation (FISHER 1930, 1935; HUTCHINSON 1931). The best that can be said for this hypothesis is that none of the available evidence provides factual support for it.[1] The results of crossbreeding suggest that factors which interfere with the "standard" expression of polydactylous breeds, viz., the bilaterally five-toed condition, are widely distributed among non-polydactylous stocks. These are factors which may lead to partial or complete suppression or to an exaggeration of the "standard" condition. They have

[1] It may be remarked in passing that the general hypothesis of dominance factors, as it applies to poultry, would merely shift the question of why so many mutants of fowl, compared with other animals, are dominant to that why *partial* dominance is unusually frequent.

presumably been removed, to a large extent but not entirely, in the production of polydactylous breeds. In non-polydactylous stocks these factors may provide added assurance for normal growth and development of the limb fields. This would explain their occurrence in all non-polydactylous stocks which have been tested. All results of crossbreeding experiments indicate that those factors which tend to exaggerate the polydactylous condition are much rarer in non-polydactylous stocks than those which have a partial or complete suppressing effect.

WARREN (1941) described a new type of polydactylism of fowl, known as "duplicate," which arose by mutation in a flock of White Leghorn fowl. The gene for duplicate appears to be an allele of that for polydactylism. The heterozygous expression of the duplicate mutant tends to be more extreme than that of ordinary polydactylism. The feet frequently have six toes and the wings show striking finger duplications. The homozygous condition appears to be semi-lethal. We have produced birds of very similar appearance, with digit duplications in both extremities, by selection from outcrossed stock of Silkie-Houdan origin. The modifying factors which favor expression of polydactylism in the wings were presumably derived from the normal stock. It seems reasonable to us, therefore, to assume that the original phenotype of polydactylism was similar to that of duplicate and that in the process of standardization breeders removed by selection all those genetic factors which either tended to suppress or partially inhibit bilateral and regular expression (five toes) in the feet or which promoted excessive, and often crippling and unsightly duplication of toes as well as fingers.

We believe that the developmental nature of polydactylism is somewhat elucidated by the occurrence in polydactylous stocks of ectrodactylism (enhanced by insulin treatment) and of malformations of the radius. Some cases of ectrodactylism are illustrated in plate II. These are from insulin experiments, but similar specimens are found, if more rarely, in untreated embryos. The first and fourth toe are most commonly absent in ectrodactylism. Particular attention is called to cases with duplication of the second toe, partial fusion of the second and third toe and rudimentary formation of the third toe (plate II). These instances certainly give weight to BOND'S (1926) statement that the factor for polydactylism "may influence all the digits."

A variety of abnormalities of the radius were observed in "duplicate"fowl and among polydactylous chickens from a family in which we had selected for the expression of hyperdactylism in the hand. They will be described in detail elsewhere. Suffice it to say here that these malformations range from complete or partial absence of the radius to its partial or even complete duplication.

Our observations on ectrodactylism and on radius abnormalities indicate that the effect of the gene is not merely one on the hallux, though it may usually be limited to it, nor that it is one which is solely responsible for splitting and excessive development of the first toe. GRÖNBERG (1894) suggested that polydactylism of fowl may represent an incomplete duplication of the whole foot. BRAUS (1908), on the basis of the material of KAUFMANN-WOLF (1908),

has demonstrated, convincingly I think, from the mirror-image symmetry relations of at least certain cases of polydactylism that they are instances of abortive duplication of the whole foot. Similar observations by GABRIEL (1946) led him to think in terms of an abortive duplication of the limb field. We believe, however, that such explanations are too narrow in view of the evidence from ectrodactylism and abnormalities of the radius. We believe the evidence, taken as a whole, indicates that the gene for polydactylism is responsible for an interference with the proper organization of the limb field. Factors of residual heredity, probably acting through lateral and local differential growth rates, and the presence of one or two genes for polydactylism, presumably decide whether this disturbance of the limb field will lead to ectrodactylism, complete or unilateral suppression of polydactylism, hyperphalangism, the bilateral presence of five toes, excessive forms of digit duplication in one or both pairs of extremities, and such abnormalities as lack or duplication of the radius. It may be supposed similarly that external agencies, such as low temperature, colchicine and insulin, also produce their suppressing or modifying effects (or, in the case of colchicine, even occasional duplication) by upsetting the proper organization of the limb field. The same conclusion applies presumably to the occasional appearance of polydactylism in non-polydactylous embryos as a result of insulin treatment (LANDAUER 1947). The developmental nature of these "disturbances" of the limb field remains to be discovered.

The concept of "modifying" genes, as it has been developed in the preceding pages, reveals the integrative interaction between the mutant gene and the specific developmental potentialities of the embryonic field in which the gene takes effect.

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SUMMARY

Observations on polydactylous fowl of outbred and pure-bred origin led to the following conclusions.

1. Heterodactylism was usually a heterozygous expression of polydactylism, but occurred as a rare variant among homozygotes. In unselected stocks sinistral heterodactylism was much more common than the unilateral dextral expression.

2. The incidence of sinistral heterodactylism could be raised by selection.

3. Dextral heterodactylism gave less response to selection than the sinistral form, but *inter se* matings of parents with dextral heterodactylism produced significantly more progeny of their own kind than any other type of mating.

4. Bilateral polyphalangism responded readily to selection, but uncomplicated forms of this variant were usually, perhaps always, heterozygous. In homozygotes polyphalangism tended to show some degree of duplication of the first toe.

5. The incidence of bilaterally five-toed and bilaterally six-toed chicks could be increased by selection. In our outbred stock the bilaterally five-toed condition was a heterozygous phenotype, homozygotes having more than five toes on each foot, whereas in pure-bred Dorkings the five-toed condition is the standard expression of homozygotes.

6. Non-polydactylous chicks occurred as rare variants in homozygous polydactylous stock. Among the progenies of heterozygous fowl the incidence of exceptional non-polydactylous chicks varied with the phenotype of the parents, matings of heterodactylous chickens producing significantly more non-polydactylous offspring than matings of bilaterally hyperdactylous animals.

7. Chicks with ectrodactylism are a rare but significant feature of polydactylous stocks.

8. The injection of insulin led to phenotypic modifications of polydactylism. The period of maximum response was between 72 and 96 hours of incubation. Insulin treatment may lead to an increased incidence of heterodactylism or hyperphalangism or the complete suppression of polydactylism; it also raised the frequency with which ectrodactylism occurred. The particular type of phenotypic response to insulin varied with the tendency of different stocks to produce one or another kind of aberrant phenotype. In embryos of outbred stocks, whether heterozygous or homozygous, insulin produced a much greater shift in phenotypic expression than in those with the standardized genotype of polydactylous breeds.

9. Hyperdactylism of the upper extremity was only found in animals which were homozygous for polydactylism. As is true for the toes, sinistral heterodactylism of the fingers was much more common than dextral heterodactylism. Heterodactylism as well as homodactylism of the fingers were only found in animals with bilateral hyperdactylism of the toes.

10. In "duplicate" and polydactylous stocks hyperdactylism of the hand is sometimes associated with abnormalities of the radius, ranging from entire lack to complete duplication.

11. It appears that in the production of five-toed breeds factors which have an inhibiting or suppressing and others which have an exaggerating effect on phenotypic expression of polydactylism have been removed by selection.

12. It is believed that the gene for polydactylism produces a disturbance in the organization of the limb field and that the phenotypic variations of expression are mediated by (naturally occurring or experimentally induced) lateral asymmetries of or local differentials in growth intensity of the regions concerned.

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GENETICS OF NATURAL POPULATIONS. XVI. ALTITUDINAL AND SEASONAL CHANGES PRODUCED BY NATURAL SELECTION IN CERTAIN POPULATIONS OF DROSOPHILA PSEUDOOBSCURA AND DROSOPHILA PERSIMILIS

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INTRODUCTION

PREVIOUS work has shown that populations of *Drosophila pseudoobscura* which inhabit localities only a few miles apart may differ in the incidence of certain genetic variants. Furthermore, the composition of a population may change with time; the changes are either cyclic, paralleling the annual succession of seasons, or exhibit more sustained trends which may last for several years. Thus, the populations of three localities on Mount San Jacinto, in California, differ very appreciably in the incidence of gene arrangements in the third chromosome. The localities in question lie at different elevations, but the distances between them are not over 15 miles. In the populations of two of these three localities, the frequencies of some gene arrangements undergo cyclic seasonal changes. In the third locality, no seasonal changes have been recorded; instead, between the years 1939 and 1946, the population has shown a steady increase in the frequency of a certain gene arrangement, and decreases in the frequencies of two other arrangements (DOBZHANSKY 1943, 1947a, b). At least the cyclic changes are caused by natural selection, which favors possessors of certain chromosomal types at some seasons, and of other types at other seasons. Indeed, some of the changes which take place in nature can be reproduced experimentally in "population cages." The experiments disclose that individuals heterozygous for two different gene arrangements possess the highest adaptive values, while the corresponding homozygotes are discriminated against to different extents. The superiority of the heterozygotes results in a balanced polymorphism, which acts as a sensitive mechanism enabling the species to adapt itself very rapidly to variations in the environment (WRIGHT and DOBZHANSKY 1946, DOBZHANSKY 1947a, c).

The present article reports observations on altitudinal gradients and on seasonal changes in the frequencies of gene arrangements in the third chromosome of *Drosophila pseudoobscura* and *Drosophila persimilis* living in and near Yosemite National Park, in the Sierra Nevada of California. This region lies some 300 miles north of Mount San Jacinto. Experiments on the behavior of the Sierran chromosomes in population cages will be reported in another publication. The purpose of these observations and experiments has been not only to show whether the phenomena previously observed on Mount San

Jacinto occur also in a geographically remote group of populations of the same species, and in a related but distinct species, *Drosophila persimilis*. A more urgent problem is that of the causation of these phenomena. The same chromosomal types (gene arrangements) occur in the populations of San Jacinto and of the Sierra, albeit with different frequencies. Are the genic contents and the adaptive properties of these chromosomal types alike or different in the two regions? Is the chromosomal polymorphism performing the same biological function in both regions and in both species, or is this adaptive mechanism versatile enough to serve different functions in different places?

MATERIAL AND TECHNIQUE

Samples of the populations were collected in 1946 and 1947 in several localities on the long western slope of the Sierra Nevada, and in a single locality (Timberline) on the abrupt eastern slope (fig. 1). The collecting localities are arranged approximately in a west-east transect across the Sierra Nevada, from the foothills of the western slope to the Subalpine Zone. For a description of the natural environments of the Sierra Nevada in the Yosemite Park region,

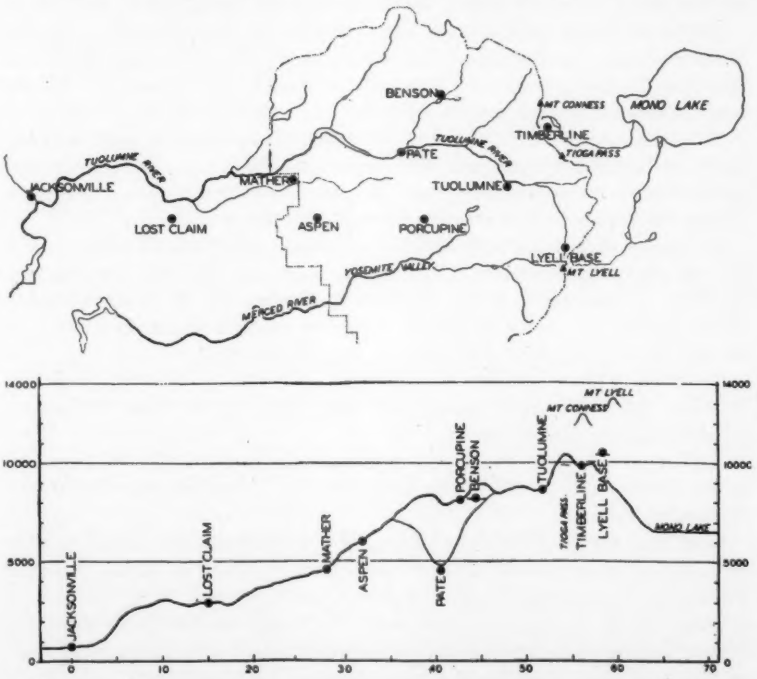

FIGURE 1.—A map and a schematic cross-section of the Sierra Nevada in the Yosemite region, showing the position of the collecting localities. Horizontal distances in miles, vertical distances in feet. The dotted line on the map is the boundary of Yosemite National Park.

see GRINELL and STORER (1924), and CLAUSEN, KECK, and HIESEY (1940). A brief description of the collecting localities follows. The first seven localities on the list have been visited repeatedly, while a single collection was made in the remaining three.

Jacksonville. Flies were collected in the oak-pine grove across the Woods Creek from the village of Jacksonville, not far from the confluence of the Woods Creek with the Tuolumne River. Elevation 850 feet, Upper Sonoran life zone. The commonest species is *D. pseudoobscura; D. persimilis* is rare except in spring and in autumn, *D. azteca* rare at all times. The Drosophila population is abundant in spring and in autumn, and contracts during the hot period of the summer. Although no collecting has been attempted in winter, it seems likely that the flies may be breeding throughout the year (See p. 174).

Lost Claim. Traps were exposed in and near the Lost Claim Campground of the U. S. Forest Service. Elevation about 3000 feet, lower part of the Transition life zone. *D. azteca* is the commonest species, followed by *D. pseudoobscura. D. persimilis* is not frequent, but in September of 1947 it proved to be more common than *D. pseudoobscura.* Winters in this and all following localities are too severe to permit uninterrupted breeding of Drosophila.

Mather. The collecting grounds were located near the experimental garden of the Carnegie Institution of Washington. Elevation 4600 feet, Transition life zone. All three species of Drosophila named above are common. The fly population is large throughout the summer.

Aspen. Luxuriant forest of the upper part of Transition life zone, near the Aspen Valley Ranger Station, elevation about 6200 feet. The commonest species are *D. occidentalis, D. pseudoobscura, D. persimilis,* and *D. pinicola. D. azteca* is infrequent but always present in collections.

Porcupine. Collections were made in the vicinity of Porcupine Flat Campground. Elevation 8000 feet, Canadian life Zone. In this and the following localities, *D. persimilis* is the commonest species, but *D. pseudoobscura* is always present. *D. azteca* is absent. Adequate samples of the fly population can be collected only in mid-summer, in July and August.

Tuolumne. Forest near the western end of Tuolumne Meadow, near the Fairview Dome. Elevation about 8600 feet, Canadian life zone (Hudsonian according to Grinell and Storer).

Timberline. Flies were collected in the neighborhood of the experimental garden of the Carnegie Institution of Washington. Elevation about 9900 feet, Hudsonian life zone.

Pate. Pine-oak forest in Pate Valley, on the bottom of the Grand Canyon of Tuolumne River. Elevation about 4200 feet. The flora of the lower part of Transition life zone penetrates along the Canyon bottom far to the east, in the heart of the Sierra. In August 1947, this locality contained an enormous Drosophila population, which consisted of *D. azteca,* with an admixture of *D. persimilis.* A single specimen of *D. pseudoobscura* was also found.

Benson. Mixed forest at the northern end of Benson Lake, elevation slightly above 8000 feet. In August 1947, a fairly large population of *D. persimilis* was found in this locality.

Lyell Base. The collection was made in late July, 1947, at the upper limit of forest vegetation near the Upper Base Camp on Mount Lyell. Elevation about 10,500 feet. Hudsonian life zone. Some *D. persimilis*, *D. occidentalis*, and a single *D. pseudoobscura* were found.

Cups with fermenting banana mash were used to attract the flies (see DOBZHANSKY and EPLING 1944 for details of the collecting technique and for information on the ecology of the flies). Whenever large numbers of flies were collected, only females were used for investigation. They were placed singly in culture bottles, and, when fully grown larvae appeared, the salivary glands of a single larva per culture were stained in acetic orcein. Examination of the chromosomes of a single larva permitted determination of the species to which the mother and the father of the larva belonged, as well as recording of the gene arrangement in two third chromosomes of the larva (one chromosome derived from the mother, and the other from the male which had inseminated the female before the latter was captured). When collections yielded small numbers of flies, males as well as females were used for examination of the chromosomes. The males were outcrossed singly to laboratory females of known species and chromosomal constitution. The salivary gland chromosomes were then examined in six larvae in the offspring of each male. Since some wild males are homozygous, and others are heterozygous, for the gene arrangement in the third chromosome, the six larvae examined in the offspring of a wild male showed either one or two types of chromosome structure. When two types are detected, the inference is clearly that the wild parent was a heterozygote. When all six larvae are alike in constitution, the probability is thirty-one out of thirty-two chances that the male was a structural homozygote. The failure to detect some heterozygotes (one in every 32) is not important for our present purpose, because the statistics considered in this paper deal with gametic, and not zygotic, frequencies of various gene arrangements in the chromosomes of the populations studied.

VARIATION IN THE GENE ARRANGEMENT IN THE THIRD CHROMOSOME

The populations of both *D. pseudoobscura* and *D. persimilis* which inhabit the Yosemite region of the Sierra Nevada are very variable with respect to the gene arrangement in the third chromosome. Seven different arrangements occur in the populations of *D. pseudoobscura*. Three chromosomal types, namely Standard (henceforth abbreviated ST), Arrowhead (AR), and Chiricahua (CH) are common in all localities. The Tree Line (TL) gene arrangement is fairly common, and Santa Cruz (SC), Olympic (OL), and Pikes Peak (PP) are rare. For descriptions and drawings of all these chromosomal types, see DOBZHANSKY 1944.

In *D. persimilis*, one gene arrangement, denoted as Whitney (WT), is the commonest throughout the Yosemite region of the Sierra. Three others, namely Standard (ST), Klamath (KL), and Mendocino (MD), are fairly common, and Sequoia (SE) is rare. A sixth gene arrangement, not previously known, has been found in a third chromosome of a single female from Mather.

This new arrangement is derived by single inversion from the ST one. The inversion, an unusually long one, involves most of the euchromatic part of the third chromosome, from section 64 B or C to section 80 C of the standard map of this chromosome. Its proximal break (in 64) may conceivably coincide with that in the inversion by which the Cuernavaca arrangement is derived from the Santa Cruz gene arrangement (DOBZHANSKY 1944, p. 65), but the distal break, in section 80 C, falls in a place in which no other natural breaks have been observed either in *D. persimilis* or in *D. pseudoobscura*. Since the new gene arrangement (which may be referred to as "Mather") has been found in only one out of 760 chromosomes of D. *persimilis* from the same neighborhood, it is probable that the Mather arrangement arose by mutation in the near ancestry of the fly in question. It may also be a chromosomal type which occurs in only a very small region in the vicinity of Mather. It is derived by inversion from the ST gene arrangement, and not from WT which is much more common in this region. However, ST chromosomes occur frequently enough in *D. persimilis* to make the conjecture of a recent origin of the Mather gene arrangement not improbable.

SEASONAL CHANGES IN POPULATIONS OF DROSOPHILA PSEUDOOBSCURA

The frequencies of gene arrangement found in the samples of *Drosophila pseudoobscura* taken in various localities are reported in tables 1–5. Examination of these tables suggests that in some, but not in all, localities the composition of the populations underwent changes during the period of observation.

TABLE 1

Frequency (in percent) of different gene arrangements in Drosophila pseudoobscura at Jacksonville.

DATE OF COLLECTING	ST	AR	CH	TL	SC	OL	PP	n
June 18, '46	42.5	25.0	24.0	7.5	1.0	—	—	200
July 20–21, '46	46.3	19.4	14.9	9.7	6.0	0.7	3.0	134
Apr. 13, '47	46.0	40.0	8.0	2.0	—	2.0	2.0	50
May 15, '47	47.6	25.4	12.9	5.2	6.5	1.2	1.2	248
June 20–21, '47	47.5	25.0	14.5	7.5	4.0	1.5	—	200
July 31, '47	45.1	24.7	14.4	8.9	5.4	1.5	—	202
Sept. 7, '47	46.4	22.3	16.1	12.5	2.7	—	—	112

The samples taken at Jacksonville (table 1) appear to be rather uniform in composition. Only one sample, taken on April 13, 1947, stands out among others because of containing too many AR, and too few CH and TL, compared with other samples. This sample is, however, rather small. The uniformity of the Jacksonville samples has been tested by computing a chi-square; for this computation, the chromosomes were divided in three classes, namely those with ST, those with AR , and those with other gene arrangements. The figures for CH, TL, SC, OL, and PP chromosomes were combined because in most samples these chromosomes are too rare to make the computation of chi-

squares free of complications. Furthermore, the ST and AR chromosomes vary in frequency from month to month in some localities (see below), while other types show no systematic changes in frequencies. For the Jacksonville samples, the chi-square proved to be 14.09, which, for 12 degrees of freedom corresponds to a probability level of about 0.3. No significant seasonal changes in the composition of the Jackonsville population are, thus, indicated by our data (See note on p. 174).

TABLE 2

Frequency (in percent) of different gene arrangements in Drosophila pseudoobscura at Lost Claim.

DATE OF COLLECTING	ST	AR	CH	TL	SC	OL	PP	n
June 24, '46	35.8	35.8	15.7	9.0	1.5	—	2.2	134
Aug. 11, '46	37.1	32.3	18.7	7.3	3.2	1.6	—	124
May 15, '47	35.2	45.7	11.3	5.6	0.7	1.4	—	142
June 20, '47	44.5	35.6	11.6	4.1	2.7	0.7	0.7	146
Aug. 6, '47	48.5	27.3	13.4	5.7	3.6	1.5	—	194
Sept. 9, '47	40.0	30.0	20.0	—	10.0	—	—	20

TABLE 3

Frequency (in percent) of different gene arrangements in Drosophila pseudoobscura at Mather.

DATE OF COLLECTING	ST	AR	CH	TL	SC	OL	PP	n
July–Aug. '45	35.7	35.7	17.2	10.4	0.6	0.3	—	308
June 26, '46	21.9	42.7	20.2	11.2	1.7	1.7	0.6	178
Aug. 10, '46	39.9	30.4	13.9	10.1	3.2	2.5	—	158
May 15, '47	25.7	47.1	18.6	4.3	—	4.3	—	140
June 22–26, '47	29.5	42.6	19.7	5.7	1.6	0.8	—	122
July 25, '47	27.9	41.3	21.1	6.7	1.9	—	1.0	104
July 26, '47	31.5	36.5	20.0	9.0	1.5	0.5	1.0	200
Sept. 1–12, '47	35.8	31.2	20.8	7.5	3.3	0.4	0.8	240

The samples obtained at Lost Claim (table 2) are heterogeneous. It may be noted that the fairly large samples obtained in May, June, and August of 1947 show a progressive increase in the frequencies of ST, and a decrease in AR, chromosomes. The September sample suggests a reversal of this trend, but this sample is small. The chi-square for the four samples taken in 1947 is 13.99, which, for 6 degrees of freedom, has a probability of about 0.03. The heterogeneity of these samples is, thus, statistically fairly significant. The two samples taken in 1946 show fewer ST chromosomes than samples taken in corresponding months in 1947 do, but the differences are not reliable. It seems, then, that, at least in 1947, the frequencies of ST chromosomes waxed, and those of AR waned, as the summer progressed.

Similar seasonal changes in the incidence of ST and AR chromosomes occur also at Mather (table 3). In 1946, the frequency of ST chromosomes almost doubled between June and August, while AR and CH chromosomes became less frequent; this change is statistically quite significant ($\chi^2 = 13.05$, df = 2, P = 0.002). In 1947, the changes occurred ostensibly in the same direction as in 1946, but they were less pronounced and did not involve the CH chromosomes. The chi-square for 1947 is 12.64, which has a probability of 0.13 for

TABLE 4

Frequency (in percent) of different gene arrangements in Drosophila pseudoobscura at Aspen Valley.

DATE OF COLLECTIHG	ST	AR	CH	TL	SC	OL	PP	n
July 6–9, '46	17.5	53.2	18.3	9.5	0.8	0.8	—	126
June 19–25, '47	20.6	46.0	15.1	12.7	3.2	2.4	—	126
July 29, '47	31.3	44.7	14.0	9.3	0.6	—	—	150
Aug. 31–Sept. 11, '47	35.5	26.3	19.7	11.8	1.3	3.9	1.3	76

TABLE 5

Numbers of chromosomes with different gene arrangements in Drosophila pseudoobscura living at high elevations.

LOCALITY	DATE OF COLLECTING	ST	AR	CH	TL	SC	OL	n
Porcupine	July 13–14, '46	1	3	1	1	—	—	6
Porcupine	July 11–17, '47	5	14	10	2	1	—	32
Tuolumne	July 23–25, '46	5	3	1	1	—	—	10
Tuolumne	July 15–16, '47	3	33	16	3	1	2	58
Tuolumne	Aug. 15–16, '47	1	9	1	3	—	—	14
Pate Valley	Aug. 25, '47	1	—	—	1	-	—	2
Benson Lake	Aug. 23–24, '47	—	3	1	1	1	—	6
Timberline	July 24, '46	1	1	1	—	—	1	4
Lyell Base	July 22, '47	—	4	1	—	1	—	6

8 degrees of freedom, and thus is not significant. However, since the change in the Mather locality in 1947 occurred in the same direction as in 1946, and since, furthermore, they agree with those observed at Lost Claim and at Aspen, it is reasonable to take the Mather data for 1947 at their face value.

The samples from Aspen Valley (table 4) suggest that the frequency of ST chromosomes has increased, and that of AR chromosomes has decreased between late June and early September of 1947. This change is statistically significant ($\chi^2 = 14.57$, df = 4, P = 0.006). Only one sample was taken at Aspen in early July of 1946, and this sample contains even fewer ST, and more AR, chromosomes than the sample taken in late June 1947; the difference is, however, not significant.

Drosophila pseudoobscura is not common at elevations higher than that of Aspen Valley. The numbers of chromosomes examined in the populations of the higher localities are, therefore, small (table 5). Furthermore, the duration of the season when flies can be collected in sufficient numbers in the Canadian and Hudsonian life zones of the Sierra Nevada is apparently less than two months per year. It is not surprising, then, that no significant seasonal changes are detected in the material from Porcupine and Tuolumne populations. But the failure to detect such changes does not prove that they do not occur.

Figure 2 summarizes graphically the data on seasonal changes in the frequencies of the gene arrangements in the Sierra Nevada. At Lost Claim, Ma-

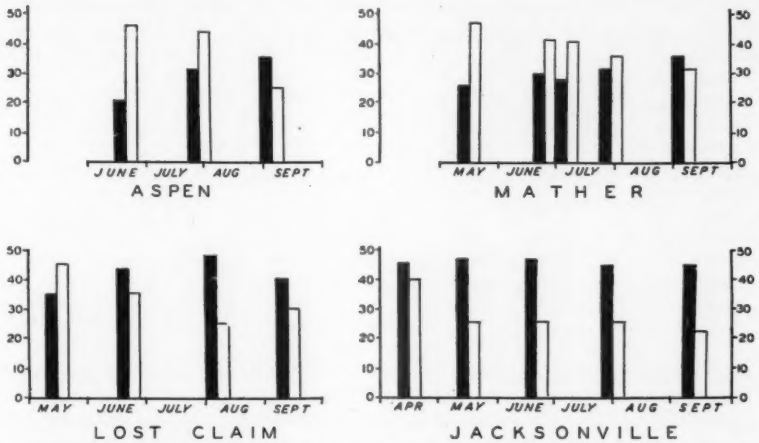

FIGURE 2.—Frequencies, in percent, of Standard (black columns) and of Arrowhead (white columns) chromosomes in *Drosophila pseudoobscura* in samples taken in different months of 1947 in five localities in the Sierra Nevada.

ther, and Aspen, the ST chromosomes become more and more frequent as the summer progresses, and appear to reach their highest annual frequencies in August and September. AR chromosomes are, on the contrary, commonest early in the season (May and June), their frequencies diminish during the summer, and reach minimal values in September. CH and other chromosomal types show no consistent seasonal trends. The cyclic changes in the proportions of ST and AR presumably continue during hibernation, when AR chromosomes must increase in frequency at the expense of ST.

A comparison of the seasonal changes in the Sierra with those on San Jacinto (DOBZHANSKY 1943, 1947a) discloses profound differences between them. In the Sierra, the changes affect the frequencies of ST and AR chromosomes, while CH are not involved; on San Jacinto, the frequencies of ST and CH change, while those of AR remain relatively (though not completely) constant. Furthermore, no changes take place during autumn and winter, on San Ja-

cinto, and the changes that take place during spring and early summer are reversed in mid-summer. In the Sierra, the direction of the changes appears to be constant during the entire season when the flies can be collected, and the reversal takes place presumably during hibernation.

It is also remarkable that no significant seasonal changes have been recorded in the population of Jacksonville, the lowest in elevation among the localities studied in the Sierra. Figure 2 suggests that the seasonal changes are greatest in the highest of the adequately studied localities (Aspen), become less pronounced at Mather and Lost Claim, and disappear at Jacksonville, in the Sierran foothills. Although such a relation between the elevation

TABLE 6

Frequency (in percent) of different gene arrangements in Drosophila persimilis at Mather.

DATE OF COLLECTING	WH	ST	KL	MD	SE	new	n
July–Aug. '45	69.7	8.6	15.2	6.1	0.5	—	198
June 26, '46	72.9	11.4	11.4	2.9	1.4	—	70
Aug. 10, '46	81.0	10.4	8.6	—	—	—	58
May 15, '47	72.9	4.2	14.6	8.3	—	—	96
June 22–26, '47	79.5	10.3	6.4	2.6	—	1.3	78
July 5, '47	75.0	14.3	7.2	3.6	—	—	28
July 26, '47	87.5	6.3	3.1	3.1	—	—	32
Sept. 1–12, '47	82.0	9.0	4.0	4.5	0.5	—	200

and the amplitude of the changes in the Sierra can not be considered proven, the situation in the Sierra is clearly different from that on San Jacinto. No seasonal changes have been found in the highest of the three localities (Keen Camp) on Mount San Jacinto, while lower localities do show changes. The converse is true in the Sierra.

Whether the populations of the Sierran localities change not only from season to season but also from year to year, can not be decided on the basis of the available information. The differences between the samples taken in the same locality during the same months of the two successive years, 1946 and 1947, are not very significant statistically. This negative finding, of course, does not exclude the possibility that variations from year to year, nevertheless, do occur. It may be noted that the year 1947 was much drier than 1946 and 1945. The relative frequencies of the species *D. pseudoobscura*, *D. persimilis*, and *D. azteca* in the same localities were rather strikingly different in those years; *D. pseudoobscura* was more common at higher elevations in 1947 than earlier. Some indications exist that ST chromosomes were less, and AR more, common in 1946 than in 1947.

LACK OF SIGNIFICANT SEASONAL CHANGES IN DROSOPHILA PERSIMILIS

The available data on the incidence gene of various arrangements in different samples of *D. persimilis* are summarized in tables 6–9. Inspection of these

data discloses no striking changes in the composition of *D. persimilis* populations from season to season, but a statistical analysis leads to uncertain results.

Among the samples taken in 1946 at Mather (table 6), the lowest frequency of WT chromosomes was found in May (about 73 percent), and the highest in late July (87.5) and in September (82 percent). An ostensible increase in the frequency of WT took place also in 1946 between June (73 percent) and August (81 precent). To test the heterogeneity of the samples taken at Mather and

TABLE 7

Frequency (in percent) of different gene arrangements in Drosophila persimilis at Aspen Valley.

DATE OF COLLECTING	WH	ST	KL	MD	n
July 6–9, '46	68.5	11.1	14.8	5.6	108
June 19–25, '47	77.2	12.0	7.6	3.3	92
July 29, '47	82.8	6.9	5.2	5.2	58
Aug. 31–Sept. 11, '47	83.9	8.0	3.6	4.5	112

TABLE 8

Frequency (in percent) of different gene arrangements in Drosophila persimilis living at high elevations.

LOCALITY	DATE OF COLLECTING	WH	ST	KL	MD	SE	n
Porcupine	July 13–14, '46	76.8	11.0	7.3	4.9	—	82
Porcupine	July 11–17, '47	85.1	9.1	2.5	2.5	0.8	121
Pate Valley	Aug. 25, '47	76.7	13.3	5.0	5.0	—	60
Benson Lake	Aug. 23–24, '47	88.2	9.1	2.7	—	—	110
Tuolumne	July 23–25, '46	92.5	5.0	2.5	—	—	80
Tuolumne	July 15–16, '47	90.5	4.0	2.5	3.0	—	200
Tuolumne	Aug. 15–16, '47	90.5	4.0	—	5.4	—	74
Timberline	July 24, '46	93.3	—	3.3	3.3	—	30
Timberline	Aug. 3–4, '47	90.9	6.8	—	2.3	—	44
Lyell Base	July 22, '47	87.9	8.6	3.4	—	—	58

listed in table 6, a chi-square has been computed, and proved to be equal to 12.83, which, for 7 degrees of freedom, has a probability of about 0.08. (In this and in the following computations, the chromosomes are divided in two groups, those with WT and those with all other gene arrangements.) The greatest contribution to this chi-square is, however, made by the sample collected in 1945, which contained only about 70 per cent of WT Chromosomes. The reality of the seasonal changes is, therefore, uncertain.

The samples taken at Aspen (table 7) show frequencies of WT ranging from 68.5 to 84 percent. The chi-square is 8.61, which, for 3 degrees of freedom, has a probability of 0.04; this, according to the usual convention, connotes a sig-

nificant heterogeneity. But again, as in the case of the samples from Mather this heterogeneity is caused mainly by a difference between the 1946 and 1947 samples, the former containing fewer WT chromosomes than the latter. On the other hand, the data for 1947, if taken at face value, suggest the same increase in the frequency of WT toward the end of the summer which is suggested by the Mather data.

The material from Lost Claim and from Jacksonville (table 9) is scanty, because *D. persimilis* is not common in those localities. It may, nevertheless, be noted that WT chromosomes were less frequent at Lost Claim in 1946

TABLE 9

Numbers of chromosomes with different gene arrangements in Drosophila persimilis living at low elevations.

LOCALITY	DATE OF COLLECTING	WT	ST	KL	MD	n
Jacksonville	June 18, '46	2	—	—	—	2
Jacksonville	April 13, '47	9	2	—	1	12
Jacksonville	May 15, '47	7	1	1	5	14
Jacksonville	July 31, '47	4	—	2	2	8
Jacksonville	Sept. 7, '47	14	3	4	3	24
Lost Claim	June 24, '46	11	—	6	1	18
Lost Claim	Aug. 11, '46	8	1	1	2	12
Lost Claim	May 15, '47	53	9	3	1	66
Lost Claim	June 20, '47	12	1	—	1	14
Lost Claim	Aug. 6, '47	2	—	—	—	2
Lost Claim	Sept. 9, '47	49	7	3	1	60

(63 percent) than they were in 1947 (82 percent). This agrees, then, with the indications obtained at Aspen and at Mather. No change during the summer of 1947 is suggested by the Lost Claim data. The two samples taken at Porcupine (table 10) in 1946 and 1947 also show ostensibly more WT chromosomes in 1947 than in 1946 (table 10).

To summarize: the data strongly suggest, but do not prove, that there has been an increase in the frequencies of WT chromosomes between 1946 and 1947 in the Sierra Nevada. If any seasonal changes at all were taking place in these populations during the summer of 1947, which is even less well established than are the changes between the years, the WT chromosomes increased in frequencies toward the end of the summer.

ALTITUDINAL GRADIENTS IN DROSOPHILA PSEUDOOBSCURA

The incidence of the chromosomal types in the combined samples from all localities is shown in table 10, and the data for the ST, AR, and CH gene arrangements are represented graphically in figure 3. Despite the seasonal inconstancy of the frequencies of some of the chromosomal types in certain localities (see above), the comparison of localities is legitimate. Indeed, sam-

ples of the populations have been taken during most of the season when the flies can be collected in reasonable numbers. This season is, to be sure, very short at elevations above the 7000 foot level. However, the frequencies observed during the mid-summer in all the localities are reasonably close to the all-year averages. The small samples obtained at Porcupine and at Benson are combined in table 10, and so are the samples from Timberline and from Lyell

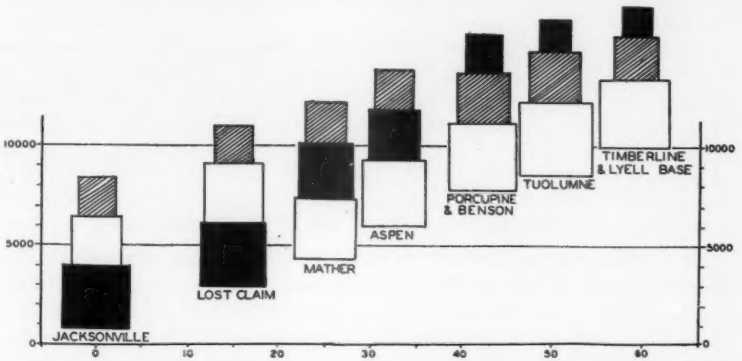

FIGURE 3.—Frequencies of Standard (black), Arrowhead (white), and Chiricahua (hatched) chromosomes in *Drosophila pseudoobscura* at different elevations in the Sierra Nevada. The areas of the squares are proportional to the frequencies of the chromosomal types. Horizontal distances in miles, vertical distances in feet. The elevation of the locality is symbolized by the position of the base of the lowermost square.

Base; the two localities of each pair lie at not very different elevations and are biotically rather similar.

The situation revealed by the data in table 10 and figure 3 is quite clear. The ST gene arrangement is commonest in the lowest locality, Jacksonville. It becomes less frequent as one ascends the slope of the Sierra Nevada, and

TABLE 10

Frequency (in percent) of different gene arrangements in Drosophila pseudoobscura in the localities along the Sierra transect.

LOCALITY	ST	AR	CH	TL	SC	OL	PP	n
Jacksonville	45.90	24.78	15.71	7.77	4.19	0.96	0.70	1146
Lost Claim	40.92	34.74	14.08	6.05	2.63	1.05	0.53	760
Mather	31.86	37.45	18.83	8.55	1.72	1.17	0.41	1450
Aspen	25.52	44.35	16.32	10.67	1.46	1.46	0.22	478
Porcupine & Benson	13.64	45.45	27.27	9.09	4.54	—	—	44
Tuolumne	10.98	54.88	21.95	8.54	1.22	2.44	—	82
Timberline & Lyell Base	10.00	50.00	20.00	—	10.0	10.0	—	10

reaches minimal values, which amount to only one quarter of its frequency at Jacksonville, above 8500 feet (Tuolumne, Timberline, and Lyell Base). AR, on the contrary, is commonest at high elevations, shows a downward gradient as one descends the Sierran slope, and reaches a minimum at Jacksonville. All other gene arrangements show no significant variations, with the possible exception of CH. The CH chromosomes seem to be more common above 7000 feet (from Porcupine to Lyell Base) than they are below that level (from Jacksonville to Aspen).

The altitudinal gradients in the Sierra are, thus, very different from those observed on Mount San Jacinto (DOBZHANSKY 1943). In the latter region, ST chromosomes are commonest, and CH least common, in the lowest locality (Andreas), while the relation is reversed in the highest locality (Keen); the frequency of AR is uniform in the localities studied. In the Sierra, ST and AR are variable, while CH is relatively constant.

ALTITUDINAL GRADIENTS IN DROSOPHILA PERSIMILIS

The data bearing on the frequencies of the chromosomal types at different elevations in the Sierra Nevada are summarized in table 11. A clear relationship is apparent: WT chromosomes are least frequent in the lowest locality, Jacksonville, and most frequent in the Subalpine zone (Tuolumne, Timberline, Lyell Base). ST, KL, and MD gene arrangement are more frequent in the lower than in the higher localities. The differences are statistically highly significant. The altitudinal gradients in *D. persimilis* seem to be more uneven than those in *D. pseudoobscura* (table 10). Thus, the frequencies of WT chromosomes seem to be about the same in all localities at mid-elevations (Lost Claim, Pate, Mather, and Aspen), and increase rapidly further up the slope. This unevenness may, however, be spurious and due to sampling errors.

TABLE 11

Frequency (in percent) of different gene arrangements in Drosophila persimilis in the localities along the Sierra transect.

LOCALITY	WT	ST	KL	MD	SE	n
Jacksonville	60.00	10.00	11.67	18.33	—	60
Lost Claim	78.49	10.47	7.56	3.49	—	172
Pate	76.67	13.33	5.00	5.00	—	60
Mather	76.45	8.82	9.61	4.61	0.40	760
Aspen	77.57	9.73	8.11	1.59	—	370
Porcupine & Benson	84.03	9.58	3.83	2.24	0.32	313
Tuolumne	90.96	4.24	1.98	2.82	—	354
Timberline & Lyell Base	90.15	6.06	2.27	1.52	—	132

An interesting detail is that the rather small sample from Pate Valley proved to be similar in composition to that from a rather remote locality, Lost Claim (see figure 1), and different from those from Porcupine and Benson, which are much nearer Pate Valley in distance. In fact, Pate Valley lies about midway

between Porcupine and Benson, but at an elevation almost 4000 feet lower than the latter two localities. On the other hand, the elevation of Pate Valley is intermediate between those of Lost Claim and Mather. Taken at face value, this fact would indicate that the frequencies of the chromosomal types in a population are determined by the altitude (and, hence, by the environment) of the locality where the population resides, rather than by the spatial proximity to other populations.

DISCUSSION

Differences in the incidence of gene arrangements in the third chromosome may occur between populations of *Drosophila pseudoobscura* which reside in localities not very far apart (in relation to the total distribution area of the species) in the same geographic region. Such "microgeographic races" may or may not be isolated from each other by migration barriers. Thus, considerable differences have been found between the populations of mountain ranges, 30 to 200 miles apart, in the Death Valley region of California (DOBZHANSKY and QUEAL 1938). The populations of adjacent ranges proved to be, on the average, about as distinct as those of ranges farther apart. The ranges are partly isolated from each other by deserts in which *D. pseudoobscura* occurs rarely, and in which the highest annual population density of the flies occurs at the season when the mountain populations are in hibernation. KOLLER (1939) found that populations of different canyons on a single mountain range vary in composition to about the same extent as do those of different ranges. The isolation of populations which inhabit different canyons is certainly less complete than that of populations on different ranges. DOBZHANSKY (1939, 1944) found differences between populations of localities only a few miles distant in Texas and in California, these localities being parts of territories presumably continuously inhabited by the flies. The differences between the populations of localities on Mount San Jacinto (DOBZHANSKY 1938), and in the Sierra Nevada (the present article), are further examples of genetic differentiation of populations of localities within a continuously inhabited region.

DOBZHANSKY and QUEAL (1938), KOLLER (1939), and DOBZHANSKY (1939) ascribed the microgeographic differentiation among populations of *D. pseudoobscura* to genetic drift in semi-isolated colonies of medium to small effective population size. That such colonies indeed drift apart in their genetic constitution has been shown by analysis of the frequencies of alleles among recessive lethals found in the third chromosomes of populations of the Death Valley and the San Jacinto regions (DOBZHANSKY and WRIGHT 1941; WRIGHT, DOBZHANSKY, and HOVANITZ 1942). But the genetic drift hypothesis is no longer tenable as far as the differentiation with respect to the gene arrangements in the third chromosome is concerned. The discovery of seasonal changes in the frequencies of gene arrangements in some localities on San Jacinto (DOBZHANSKY 1943), and the experimental reproduction of some of these changes in artificial populations (WRIGHT and DOBZHANSKY 1946; DOBZHANSKY 1947c), has shown that the carriers of different gene arrangements have different adaptive values and are subject to very high selective pressures. The differ-

ences in relative frequencies of gene arrangements between neighboring populations may, consequently, arise through, and be maintained by, natural selection. Altitudinal gradients, such as that described in the present article for the Sierran populations, also argue against the origin of microgeographic differentiation through genetic drift and in favor of its origin through natural selection. Altitudinal races arise as a response of the species to environmental differences in the habitats at different elevations. Accordingly, altitudinal races fit the definition of "ecotypes" proposed by TURESSON (1922). They are essentially comparable to the altitudinal ecotypes in plant species studied in the Sierra Nevada by CLAUSEN, KECK, and HIESEY (1940).

The ecotypes of TURESSON and of CLAUSEN, KECK, and HIESEY are, however, entities evolved in the historical process of adaptation of species to their environments, and these ecotypes appear to be rather well integrated units which undergo no appreciable changes during short time intervals. The altitudinal ecotypes in Drosophila are, on the contrary, in a constant flux. This is presumably because the length of a generation in Drosophila flies is so short that the population responds to the environmental differences which it encounters at different seasons by adaptive modifications of its genetic composition. In Drosophila, it is possible to speak of seasonal, as well as of altitudinal, ecotypes. Indeed, the same gene arrangements occur at all elevations at which the species lives, and, of course, at all seasons of the year. The differences observed at different seasons within a locality are, therefore, substantially identical with those observed between populations of different localities at different elevations. As shown above, populations of *Drosophila pseudoobscura* at lower altitudes in the Sierra, have more ST and fewer AR chromosomes than do populations of higher altitudes. Now, within some of the populations, ST chromosomes become more and AR less frequent as the season progresses and the weather becomes warmer. To state it differently, populations of high elevations acquire during the warm season the same genetic composition as populations of low elevations show during colder seasons. Vice versa, populations of lower elevation acquire during winter a resemblance to populations of higher localities during the warm part of the summer. Altitudinal and seasonal ecotypes are different aspects of the same phenomenon. Ecotypes described in plant species have evolved slowly, and, within time intervals of the order of a human lifetime, appear to be stable entities. The nature of the processes which led to their formation is a matter of inference. The selective differentials between the carriers of different gene arrangements in Drosophila are so great that natural selection forms and re-forms the ecotypes yearly.

The absence of seasonal changes in the population of the lowest of the Sierran localities, Jacksonville (figures 1 and 2), requires an explanation. Surely, the environment of this locality undergoes seasonal changes. The apparent absence of a reaction of the species to these changes may be due to either of two causes. First, since the essential property of the adaptive system of gene arrangements is that inversion heterozygotes have higher adaptive values than do inversion homozygotes, the frequencies of the gene arrangements in a population can not changed beyond certain equilibrium values (DOBZHANSKY 1947a).

It is possible, then, that the Jacksonville population exists the year round at equilibrium. This population is submarginal, since the species occurs rarely, if at all, at elevations lower than Jacksonville, in the Lower Sonoran life zone. Second, although the Jacksonville population contains the same gene arrangements as do other populations, the gene contents of the chromosomes may be different, and the seasonal changes in this environment may not elicit adaptive responses in the form of changes in the relative frequencies of the gene arrangements. This possibility is made plausible by two sets of facts. Seasonal changes on Mount San Jacinto have been recorded in two localities at lower elevations, while in the highest locality no seasonal changes occur (DOBZHANSKY 1943, 1947a,b). Unpublished data show that the chromosomes with the same gene arrangements in these localities have different adaptive properties. In *Drosophila persimilis* no seasonal changes seem to occur. The biological function of the chromosomal polymorphism in certain populations of *Drosophila pseudoobscura* is concerned in part with adaptation to seasonal changes in the environment, while in other populations of the same species, and in other species, the same or an analogous polymorphism apparently performs other adaptive functions.

Chromosomal polymorphism is extremely versatile as an adaptive mechanism. It will be shown in another publication that the gene contents of Standard, Arrowhead, and Chiricahua chromosomes from San Jacinto differ from those of the chromosomes with the same gene arrangements which occur at Mather, in the Sierra; even the chromosomes of the Piñon population on San Jacinto differ from those of the Keen population, about 15 miles away. Fly populations of different localities are adapted to their respective environments. In the present article, data are reported which show a profound difference between the altitudinal and seasonal variations on Mount San Jacinto and in the Sierra. On San Jacinto, the populations of localities at different elevations differ in the frequencies of Standard and Chiricahua, but not of Arrowhead, chromosomes. In the Sierra, altitudinal differences involve mainly Standard and Arrowhead gene arrangements, Chiricahua being nearly constant.

In the two lower localities, Andreas and Piñon, on San Jacinto, Standard chromosomes decrease and Chiricahua increase in frequency from March to June. It happens that in the population of the higher locality, Keen, in which no seasonal changes occur, the Standard and Chiricahua chromosomes are at all times about as frequent as they become during the month of June at Andreas and at Piñon. Now, the June population of Keen is expanding in numbers, while those of Piñon, and especially of Andreas, are contracting owing to excessive heat. This led to the suggestion that the seasonal changes observed at Andreas and Piñon are due to migration of the flies from Keen, or neighboring, localities, to Piñon and Andreas. This migration hypothesis is invalidated by several facts. First, the migration rates of the flies are known to be far too slow to permit movements of masses of individuals for distances of the order of ten miles in a few months (DOBZHANSKY and WRIGHT 1943, 1947). A stray individual might conceivably be transported passively by wind, but such passive transport of masses of individuals is excluded because the flies tend to be

inactive on windy days. Second, migration can not account for the increase in the frequency of Standard and the decrease of Chiricahua chromosomes which is observed at Piñon and at Andreas between June and August (DOBZHANSKY 1943, 1947a, b). Third, the maximum densities of the populations at Keen and adjacent territory are reached not in June, but in July-August. Accordingly, the maximal resemblance of the Piñon and Andreas population to that of Keen should be observed in late summers. In reality, in July-August the Piñon and Andreas populations diverge from Keen (DOBZHANSKY 1943). Fourth, the application of the migration hypothesis to the situation found in the Sierra leads to impossible results. Indeed, here it is the population of the lowest locality, Jacksonville, which shows no seasonal changes in frequencies of gene arrangements, and which contracts in numbers during July and August. Yet, the populations of the localities higher up the slope evolve in the direction of Jacksonville during the summer, when they reach highest annual densities. By analogy with San Jacinto, one would expect the higher populations to send migrants down the slope and produce changes exactly opposite to those observed. The migration hypothesis has no rational basis. All facts now available are consistent with the view that the altitudinal and seasonal variations are produced by natural selection.

A NOTE ADDED IN MARCH 1948

On February 17, 1948 PROFESSOR CARL EPLING very kindly collected a sample of Drosophila flies at Jacksonville, and sent this sample to the writer for examination. This sample, the only one in our material collected in winter, proved to be exceptionally interesting. Only 36 percent of the flies belonging to the obscura species group proved to be *Drosophila pseudoobscura*, and 64 percent were *D. persimilis*. This is the highest incidence of *D. persimilis* relative to *D. pseudoobscura* ever observed at Jacksonville. As could be inferred from its altitudinal distribution, *D. persimilis* is superior to *D. pseudoobscura* at low, and inferior at high, temperatures. Accordingly, the incidence of *D. persimilis* in the population of a locality increases during the cool seasons, and drops during the hot part of the year. Among the 172 third chromosomes of *D. persimilis* that were analyzed from the February sample, 56.4 percent had WT, 23.3 percent KL, 11.1 percent MD, and 9.3 percent had ST gene arrangements. This composition of the sample confirms the conclusion reached on the basis of less extensive data reported above (table 11), namely that the frequency of WT chromosomes is lower in the foothills than it is in the upland populations in the Sierra Nevada. Conversely, KL and MD chromosomes are much more common in the foothills than they are at higher elevations. Among the 96 chromosomes of *D. pseudoobscura* examined, 37.5 percent were ST, 34.4 percent AR, 19.8 percent CH, 4.2 percent SC, 3.1 percent TL, and 1.0 percent OL. Comparison of these figures with those listed in table 1 suggests the possibility that, although no seasonal changes in the frequencies of ST and AR chromosomes occur in the Jacksonville population from June to September, the frequency of AR may increase, and that of ST may drop during the winter months.

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SUMMARY

Seven different gene arrangements in the third chromosome occur in the populations of *Drosophila pseudoobscura* which inhabit Yosemite region of the Sierra Nevada of California. One of the gene arrangements, Standard, is most frequent at low elevations, and becomes progressively less frequent as one ascends the slope of the Sierra Nevada. Another, Arrowhead, is most frequent in the subalpine zone and decreases in frequency down the slope (see figures 1 and 3). In some of the populations, Standard chromosomes increase in frequency as the summer progresses, while Arrowhead chromosomes become less frequent (figure 2). The changes are probably reversed in winter. Thus, during the warm season, the populations of localities at higher elevations come to resemble in composition the populations of lower localities during cooler seasons.

Six gene arrangements occur in third chromosomes of the populations of *Drosophila persimilis* on the Sierra Nevada. One of the gene arrangements is described in the present article for the first time. Chromosomes with the Whitney gene arrangement are more frequent among the inhabitants of lower elevations than in those living higher up; other gene arrangements show the opposite regularity. No seasonal changes have been recorded in this species with any assurance, but there apparently has been a perceptible change in the composition of the populations between 1946 and 1947.

The seasonal and altitudinal changes are produced by natural selection.

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SPECTROPHOTOMETRIC AND CHROMATOGRAPHIC ADSORPTION ANALYSIS OF THE RED EYE PIGMENT OF DROSOPHILA MELANOGASTER

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EYE color in the wild-type of *Drosophila melanogaster* depends upon the presence of two granular pigments, a red pigment and a brown pigment. They differ in their distribution in the eye, in the time of their appearance, in their chemical and physical properties, and in being derived from different precursor substances. (For a review, see EPHRUSSI 1942, EPHRUSSI and HERALD 1944, 1945; SCHULTZ 1935; COCHRANE 1937; MAINX 1938).

Chromatographic adsorption analysis of extracts of wild-type and mutant flies begun by WALD and ALLEN (1946) and continued in the present study, have revealed that the red pigment consists of a number of closely related substances. The brown pigment, on the other hand, has so far resisted fractionation by this method. In figure 1 chromatograms of extracts of wild-type and three mutants are represented schematically in order to show the relationships of these pigments to each other. The brown pigment is represented by band No. 4. The rest of the bands belong to the red pigment series. Because of its heterogeneity the red pigment is in this paper referred to as the red pigment complex. During the course of the present work it was found that these bands fall into two groups which can be separated by means of their solubility differences: the red component, consisting of bands 1a, 1b, 2a, 2b, and 6, and the yellow component, which includes bands 3 and 5. Besides these, band S which is present in sepia flies and not in wild type flies, is included in the red pigment complex. As will be shown presently, all the members of the red pigment complex have very similar chemical and physical properties. The only ways in which they can be distinguished from each other are their different adsorption behavior, absorption spectra, and slight solubility differences.

The purpose of this study was to investigate the nature of the red pigment complex and the mode of its formation. In order to be able to analyse the pigment complex chemically, a method for the purification of at least one of its constituents had to be developed. Part I deals with the development of such a method. During the course of the work some new information was gained concerning the constitution and properties of this pigment system.

In part II the formation of the pigment complex during development is de-

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scribed and an attempt is made to analyse it in terms of the action of the genes involved.

I. NATURE OF THE PIGMENT COMPLEX

Extraction Methods

In all cases, cinnabar flies were used as the source of red pigment. In order to get enough pigment, a large number of flies had to be raised. Extractions in previous work had been made from isolated heads. It was found that the labo-

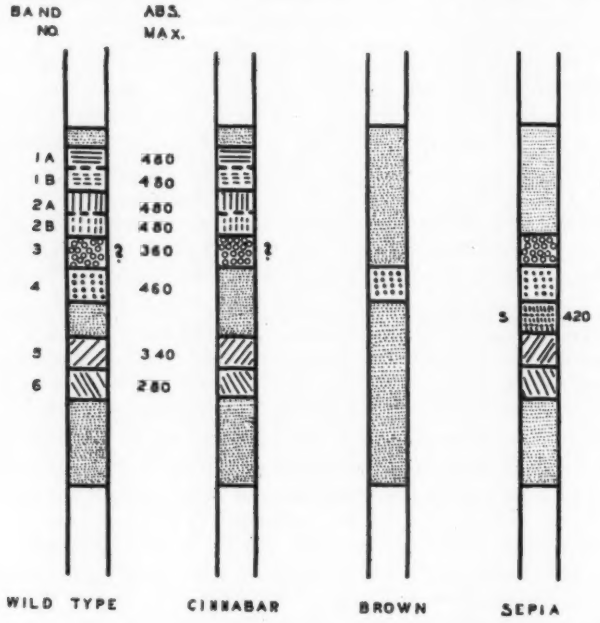

FIGURE 1.—Diagrammatic representation of chromatographic adsorption columns of extracts of wild type and three mutants. Band No. 3 has not been separated from band No. 5, but its separate existence is inferred from the study of absorption spectra. For further explanations, see text.

rious task of cutting off the heads could be avoided since body extracts yielded negligible amounts of colored substances. Thirty percent acid ethyl alcohol, pH 2 (AEA) proved to be the most useful extractant. The flies were suspended in it and ground up in a Waring mixer for about five minutes. The mash was then dialysed against the same solvent. The pigment came out readily. After about 12 hours the colored solution was poured off and fresh AEA added. This procedure was repeated until the dialysate was almost colorless and the mash inside a dirty gray color.

When small quantities of pigment were required, the Ten Broek tissue grin-

der was found to be useful for grinding up the flies. For quantitative light absorption measurements, EPHRUSSI and HERALD'S (1944) method of putting split heads into the extraction fluid was employed. Absorption measurements were carried out with a Beckman quartz spectrophotometer.

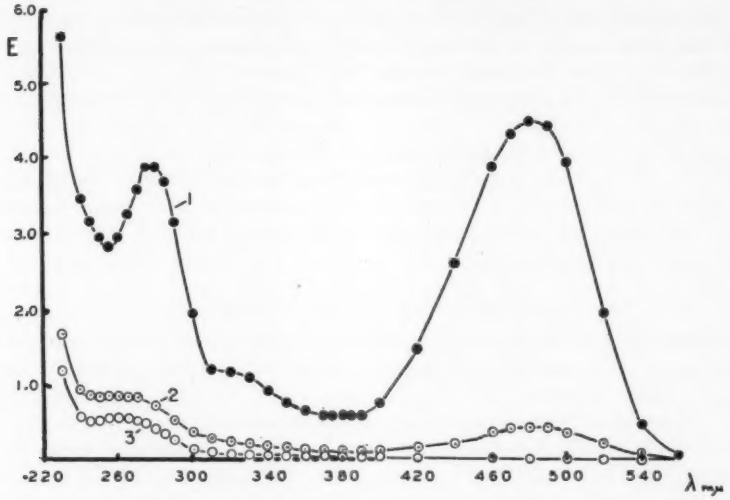

FIGURE 2a.—AEA extracts of one day old flies. 500 heads/3 cc/24 hours extraction. Curve 1: cinnabar 2: Lobe scarlet. 3: white scarlet.

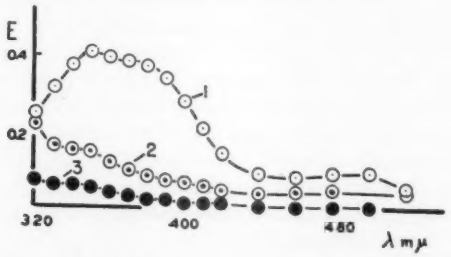

FIGURE 2b.—Methyl alcohol extracts of one day old flies. 500 heads/3 cc/12–24 hours extraction. 1: cinnabar. 2: Lobe scarlet. 3: white scarlet.

As shown by WALD and ALLEN (1946) the red and the yellow components differ markedly in their adsorption column behavior and their absorption spectra. Attempts were therefore made to separate them by means of their solubility differences. It was found that if whole heads were placed into methyl alcohol, the yellow component diffused out faster than the red. A large part of the yellow component could thus be extracted separately. Figures 2a and 2b

show extracts of the red and the yellow components from one day old flies. They also show the absorption curves of white-eye flies which lack the red pigment complex, and of Lobe scarlet flies in which because of the small size of the eyes the amount of red pigment is reduced. It can be seen that besides the red and the yellow components, the curves also differ in respect to at least one other absorption band with a maximum at 280 mμ. That this band as well as the yellow come from the eye, is shown by the presence of reduced amounts of it in the flies with reduced eye size. As will be shown later this band can be separated from the other components, and represents thus another member of the red pigment complex.

AEA extractions of vermilion and scarlet flies showed that these mutants possess the same red pigment complex as cinnabar flies. The red pigment complex in sepia flies was found to be most easily extracted with methyl alcohol. All color can be removed with this solvent from sepia vermilion flies in which the brown pigment is absent.

Chemical and Physical Properties

For the purpose of developing a purification technique some of the chemical properties of the red pigment complex had to be studied in greater detail. These will be discussed here, because besides their usefulness in purification they throw some light on the general nature of the red pigment complex.

The condition under which the red pigment complex is stable is a very important factor in working out a purification technique. Dilute AEA solution has been found to remain unchanged in the ice box for at least a week as judged by light absorption measurements. On heating the solution a brownish-red conversion product is formed whose absorption spectrum is quite different from that of the original solution. It resembles the "brown pigment" described by SCHULTZ (1935). It forms quite readily in concentrated pigment solutions on prolonged standing. Its properties are similar to those of the red pigment complex in that it is a pH indicator, and is reversibly reduced with sodium hydrosulfite. However, it is more soluble in water than the red pigment.

In alkaline solution the color of the red pigment complex fades, the speed depending on the strength of the base. In neutral solution the fading takes place very slowly. Because of the difference in the color change, the avenue of pigment breakdown seems to be quite different here from that during the formation of the brown conversion product.

In order to find out the approximate molecular weight of the red pigment complex, its diffusion constant was measured in collaboration with MR. J. ROSENBERG of the COLUMBIA UNIVERSITY Chemistry Department. Freshly dialysed AEA extracts of cinnabar flies were placed in the lower compartments of a cell of the Tiselius type (MALLETTE 1945). The upper compartments were filled with the same solvent (AEA). After establishing the liquid boundaries, the pigment was allowed to diffuse upward over a period of three days at 25°C. By that time about ten percent of the pigment had diffused across the boundary. The absorption spectra of the diffusant, diffusate, and undiffused pigment were recorded, and the diffusion constant computed from the change in

the extinction at the absorption maximum (480 mμ). From the diffusion constant the maximum molecular weight of the red component of the pigment complex was estimated to be about 300.

Chromatographic Adsorption Analysis

As described by WALD and ALLEN (1946) extracts of cinnabar heads can be separated on a talc column into at least five bands. They found that when the column is washed with water, one of these, identical with band 5 of figure 1, moves into the filtrate. They separated the other four bands of the red component by washing with dilute base.

In order to handle the relatively large amounts of pigment needed for chemical analysis and to achieve as complete a separation as possible, a long adsorption column (length 1500 mm, diameter 20 mm) was used. The adsorbent, a 5:3 mixture of talc (USP) and Celite Analytical Filter-Aid, was first washed with phosphate buffer pH 6.8, the liquid being filtered through the column by the application of suction and pressure. The rate of flow varied from ten to twenty drops per minute. A water solution of the pigment which had been adjusted to pH 6.8 with phosphate buffer was then poured into the column. It was found that bands 1 and 2 could be separated from each other by washing the column with neutral 1 M NaCl solution. This method is therefore well suited for a quantitative estimation of the major bands, because the destructive effect of alkali is avoided. To separate bands 1a from 1b, and 2a from 2b, the talc-celite column had to be washed with 1 M NaCl, containing 1 percent Na_2CO_3, adjusted to pH 9.2. At the end of three days bands 1a and 1b were completely separated from each other by white spaces of free adsorbent. Bands 2a and 2b, on the other hand, were still connected by a colored region due to the overlapping of the two bands. By that time the bands had faded considerably because of the prolonged exposure to alkali. To get the pigment back, the talc was pushed out of the column, the bands cut apart, and eluted separately with 1/100 N NaOH. The lower bands came off from the adsorbent more easily than the upper ones. The filtrates were neutralized immediately in order to prevent further destruction of the pigment. Band 2b, besides being the most easily eluted contains the largest amount of pigment of the four. According to WALD and ALLEN it constitutes approximately one half of the red component in cinnabar flies. This band was therefore chosen for further purification. In order to test its purity, it was rechromatographed on a talc column under the same conditions. It appeared there as a uniform band with a trace of a lighter region, possibly band 2a, at the top of the column. The absorption spectrum of the rechromatographed solution was identical with the original one.

It was found that a methyl alcohol solution of the red pigment complex could be fractionated partially by adsorption on alumina. Two kinds of alumina, acidic and basic, as described by WIELAND (1942) were used. On an acidic column part of the pigment comes out into the filtrate by washing the column with methyl alcohol. This portion of the pigment is richer in band 2 than the portion which remains on the column. The latter is easily eluted with water.

On a basic column, two colored bands differentiate on washing with methyl alcohol: a lower wine-red and an upper brownish-red. Both can be eluted with water.

Of the many other adsorbents tested for pigment fractionation, silicic acid (Merck) and Silene E. F. were effective in producing separation of the bands.

For the study of the red components of the sepia pigment, vermilion sepia flies were used. In the chromatogram of head extracts of these flies, three bands differentiated: an uppermost faint purple, an intense greenish-yellow, and a wide faint yellow. The two lower bands can be eluted with water, whereas for

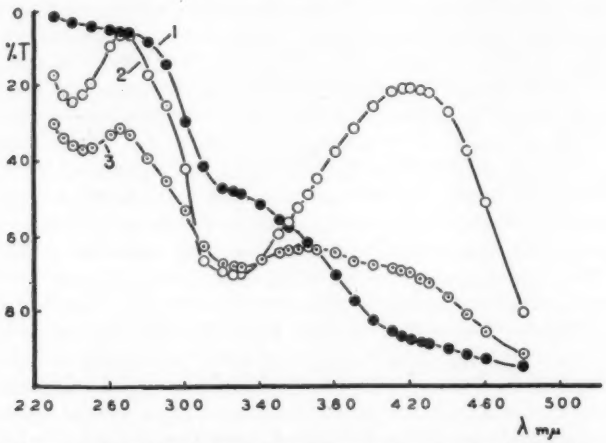

FIGURE 3.—Eluted bands from chromatogram of extracts of sepia-vermilion flies. 1: eluted band 5. 2: eluted band S. 3: eluted band 3.

the removal of the top band a stronger eluting agent, such as dilute alkali, is needed. The wide yellow band has been found to be identical with band 5 of wild-type flies (see fig. 1). Thus sepia and cinnabar flies have this component in common.

In figure 3 the absorption spectra of the three eluted bands of vermilion sepia extracts are shown. The uppermost band is apparently somewhat contaminated with the middle band. All three bands have the typical properties of the red pigment complex. For instance, on washing the column containing the three bands with sodium hydrosulfite, all three are bleached, contrary to the observation of WALD and ALLEN on sepia extracts.

Preparation of Purified Pigment

After the properties of the red pigment complex had been investigated the method illustrated in table 1 was chosen for obtaining highly purified pigment preparations of band 2b. In two of these preparations the pigment was obtained in crystalline form. To test the degree of purity of the preparation at

each step the ratio of the extinctions at 270 mμ and 480 mμ were compared. As long as there was a relative decrease in the ultraviolet absorption, impurities were being removed. The same criterion is also valid for the removal of conversion products, since they also absorb considerably in the ultraviolet. This criterion for purity can be applied until there is no more change in the absorption curve when the substance is subjected to further purification procedures.

Another test for purity is the ratio of the extinction to dry weight, or specific

TABLE I

Preparation of crystalline component 2b.

PROCEDURE	FRACTION CONTAINING DESIRED PIGMENT COMPONENT
1. Whole flies extracted with methyl alcohol until red component appears in extractant	Residue
2. Flies ground up in AEA in cold, mash dialysed in AEA until dialysant is colorless	Dialysate
3. Dialysate neutralized; alcohol removed under vacuum; solution lyophilized; powder extracted with methyl alcohol in soxlet extractor	Extract
4. Extract chromatographed 2 times on acidic alumina	Methyl alcohol eluate
5. Methyl alcohol eluate chromatographed on basic alumina	Water eluate
6. Water eluate chromatographed 2 times on talc	1/100 N NaOH eluate of band 2b.
7. Eluate neutralized and lyophilized; powder extracted with absolute ethyl alcohol in soxlet extractor	Extract
8. Pigment precipitated with ether; redissolved in large volume of isopropyl alcohol; liquid evaporated slowly on steam bath.	Crystals separate out

extinction. It was difficult to determine this ratio because the yield of final product was very small. In the largest preparation, 3.9 mg of this product were obtained from 60,000 flies. This pigment was redissolved in isopropyl alcohol and again precipitated. Of the 3.9 mg, only 1.1 mg went back in solution. The inability to redissolve all of the final pigment constitutes a serious obstacle in establishing the purity of the preparation. The recrystallization yielded 0.39 mg of which 0.10 mg could be dissolved. There was a large change in the specific extinction between the first and second crystallization. Yet the ratio of ultraviolet to visible absorption remained constant. Apparently the purification consisted of the removal of inorganic salts.

Assuming the recrystallized material to be pure, the total pigment content

of the fly was computed to be about 0.5 percent of the total wet weight. This value may appear surprisingly large. However, the eyes are relatively large, and the granules densely packed in the pigment cells, as seen in smears of the eye. The number also indicates an order of magnitude rather than an exact value since 1) the pigment is probably not quite pure, 2) the extinction coefficient is different in isopropyl alcohol from that in water, and 3) the other components have different specific extinctions from that of band 2b, presumably higher because of their more intense color.

The only chemical analysis carried out so far has been a nitrogen analysis of the eluted rechromatographed band 2b in the form of its mercury precipitate. The value for the nitrogen content of the pigment was found to be about six percent. This is considerably lower than the 19 percent recorded by LEDERER (1940). The nitrogen value found here would exclude the red pigment complex from the pterin group into which LEDERER had put it. It seems that there are so many other differences between the pterins and the red pigment that LEDERER's classification would not hold in any case.

II. FORMATION OF THE RED PIGMENT COMPLE

Description of Developmental Stages

A study of the development of the pigment system in terms of its components was begun in order to investigate the relationships of the components to each other. Quantitative estimates were made of the three main components, the red, the yellow and the colorless. The constituents of the red component were not measured separately because of the difficulty of measuring such small amounts of pigment by means of the chromatographic method. The measurements of the main components are only approximate because of the incomplete separation of the components in the double extractions.

Five stages were studied: a pupal stage just prior to the appearance of color in the eyes; a pupal stage just after appearance of the pigment (eyes yellow, bristles and wings slightly colored); a stage just before hatching of the pupa; one day old imagoes; and imagoes at least six days old. White scarlet flies of the corresponding stages were used as controls. The flies were decapitated, and the heads extracted with methyl alcohol for 12 to 24 hours, then with AEA for 24 hours. In all scarlet white experiments and in all cinnabar pupal experiments about 500 heads were extracted. For the adult cinnabar stages, fewer heads were used because of the much larger amount of pigment present.

Figures 4–7 show the absorption curves of the extracts. Extinction values are calculated for 500 heads per 3 cc. The extracts obey BEER's Law in the visible as well as in the ultraviolet region of the spectrum in the range of concentrations measured. In the ultraviolet the agreement is not quite as close as in the visible, but the deviations are negligible in comparison with the difference between various extracts.

As can be seen from the figures, the three components increase approximatly at the same rate during the same period of development of the fly. EPHRUSSI and HERALD (1945) have already measured the rate of total pigment formation by recording the absorption curves of extracts of various stages in the visible

part of the spectrum. They had come to the conclusion that the qualitative differences between their curves could be accounted for by the changes in the relative proportions of several components. They had further advanced the

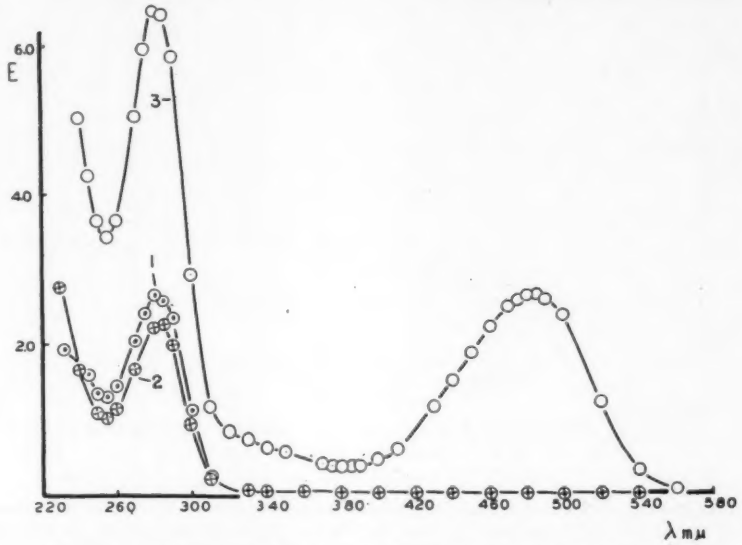

FIGURE 4a.—AEA extracts of pupal heads of cinnabar flies. 500 heads/3 cc/24 hours extraction. 1: colorless stage. 2: yellow stage. 3: orange-red stage.

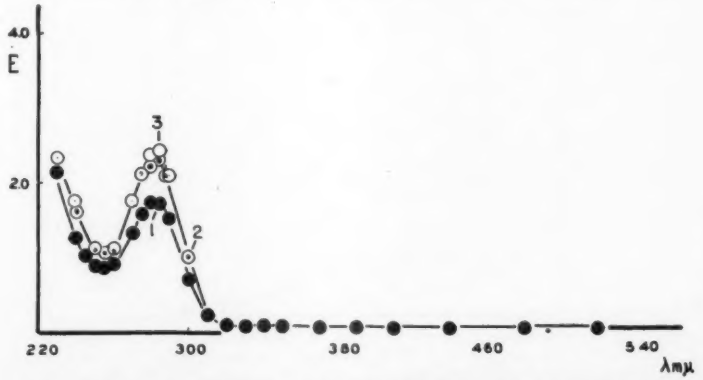

FIGURE 4b.—AEA extracts of pupal heads of white scarlet flies. 500 heads/3 cc/24 hours extraction. 1:colorless stage. 2: yellow stage. 3: orange-red stage.

working hypothesis that these components are condensation products of each other, and that pigment formation consisted of a progressive condensation of molecules from identical building blocks. On the basis of this hypothesis one

would expect to find continuous qualitative changes in the absorption spectra of the components during development. Actually the changes are only of a quantitative nature indicating that the components are formed in a parallel fashion during development.

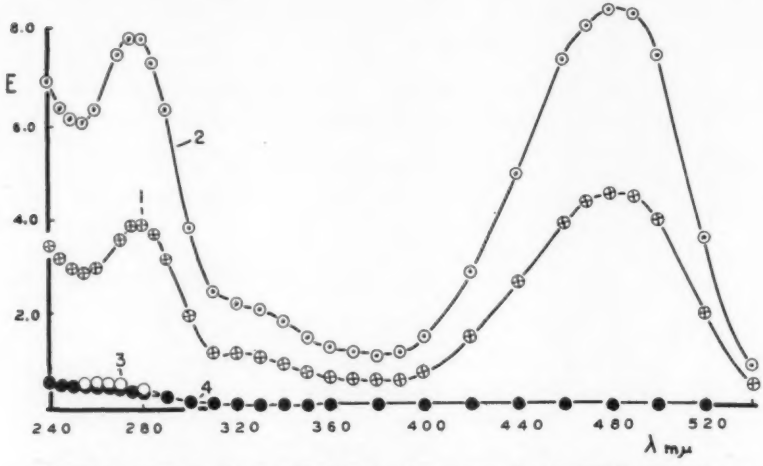

FIGURE 5.—AEA extracts of adult fly heads. Values computed for 500 heads. 1: cinnabar one day old. 2: cinnabar, six days old. 3: white scarlet, one day old. 4: white scarlet, six days old

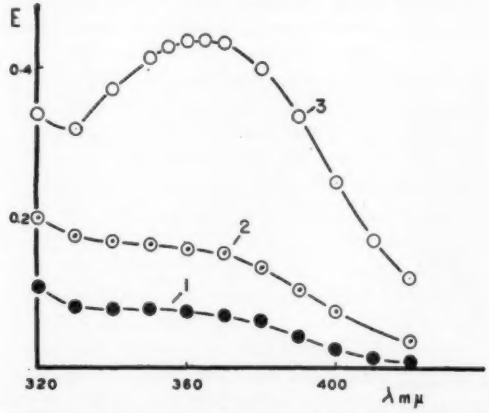

FIGURE 6a.—Methyl alcohol extract of pupal heads of cinnabar flies. 500 heads/3 cc/12–24 hours extraction. 1: colorless stage. 2: yellow stage. 3: orange-red stage.

Influence of Various Genes on Pigment Formation

As has already been pointed out, in vermilion sepia flies instead of the red component, a qualitatively different substance, the sepia component is pro-

duced. The yellow component on the other hand appears qualitatively alike in vermilion sepia and cinnabar flies. The effect of the sepia gene on the system

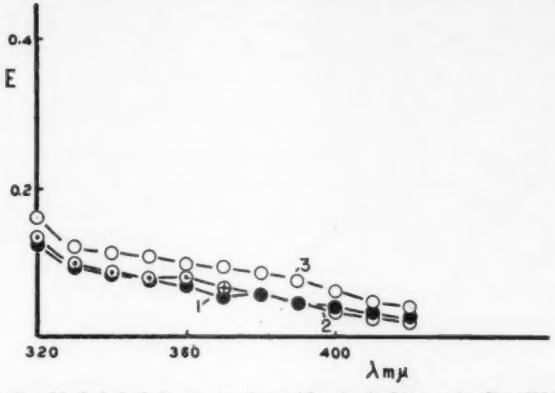

FIGURE 6b.—Methyl alcohol extracts of pupal heads of white scarlet flies. 500 heads/3 cc/12–24 hours extraction. 1: colorless stage. 2: yellow stage. 3: orange-red stage.

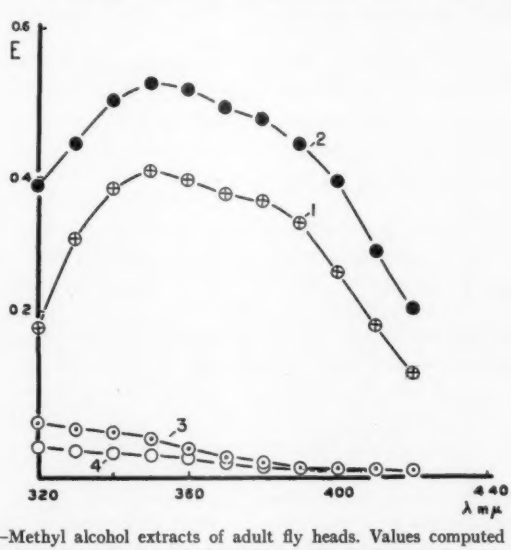

FIGURE 7.—Methyl alcohol extracts of adult fly heads. Values computed for 500 heads. 1: cinnabar, one day old. 2: cinnabar, six days old. 3: white scarlet, one day old. 4: white scarlet, six days old.

may thus be pictured as diverting the pathway of the red component without changing that of the yellow. Assuming that the two have a common precursor, the gene acts after the two components have become differentiated.

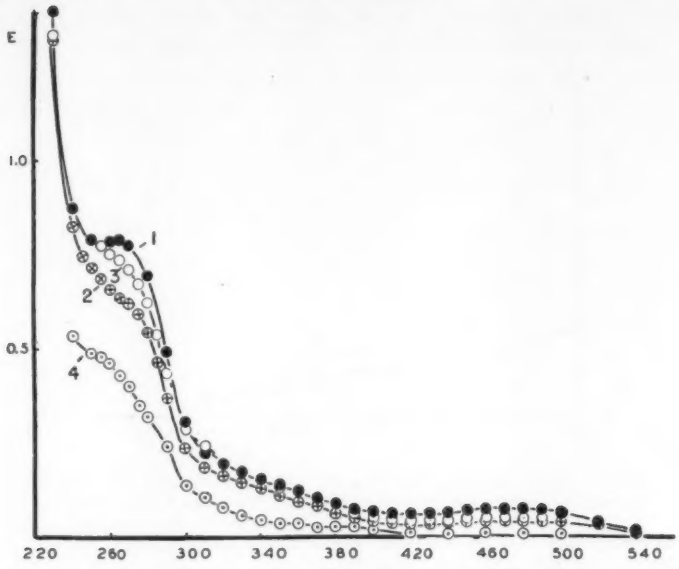

FIGURE 8a.—AEA extracts of heads of six day old flies. 500 heads/3 cc/24 hours extraction. 1: white-coral. 2: white-eosin. 3: white-apricot. 4: white scarlet.

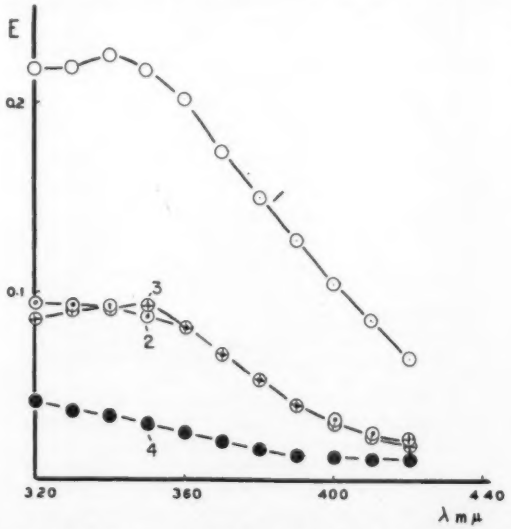

FIGURE 8b.—Methyl alcohol extracts of heads of six day old flies. 500 heads/3 cc/12–24 hours extraction. 1: white-coral. 2: white-eosin. 3: white-apricot. 4: white scarlet.

In the white allele series qualitative differences exist in the red pigment among the various members, as has already been pointed out by EPHRUSSI and HERALD (1945). Their absorption curves show that the lighter eyes have a relatively larger absorption in the 400 mμ region of the spectrum than the darker ones. In order to test whether or not these differences were caused by varying amounts of the yellow component, the relative amounts of the three pigment components were estimated in four white alleles: white-coral, white-apricot, white-eosin, and white. To avoid the presence of the brown pigment, the flies were made homozygous for cinnabar. The method of extraction was the same as that used in the study of the formation of the cinnabar pigment.

Figures 8a and 8b show the spectra of extracts of six day old flies. The overall picture as one goes from the lighter to the darker members of this allelic series is very much the same as that found in the formation of the red pigment complex in cinnabar flies: a parallel, simultaneous increase in all three components. If anything, there is relatively more of the yellow component in the darker eyes than in the lighter ones.

DISCUSSION

The further elucidation of the physiology and genetics of the red pigment complex would be simpler if its chemical nature and the chemical relationships between its constituents were known. Comparison of the red pigment complex with many of the known pigments, such as the red pigments formed in the intermediary stages of melanin formation, the pterins, the ommochromes, the cochineal pigments, and the adenochrome-urochrome group have in each case revealed discrepancies which made it impossible to include the red pigment in any of them. One will thus have to regard this pigment complex as a hitherto unknown group of substances.

That the constituents of the red pigment complex must be closely related is shown by their very similar chemical and physical properties. So far they have resisted attempts at interconversions. They are not tautomeric forms, as can be shown by readsorption of single bands at various pH's. The most likely hypothesis, at least for the members of the red component, is that they are similar compounds differing in groups which have little to do with the light absorption properties of the substances. One cannot be sure that these components exist separately in the living eye. No gene has been found yet which affects members of the red component separately. That they are not produced by the action of the talc can be shown by their separation on other adsorbents. Since the red pigment seems to be a quinone-like substance, and since quinones have a tendency to interact with other substances in solution, one always has to consider the possibility of their differentiation after extraction.

Finally it may be pointed out that it is not surprising to find pigments in the Drosophila eye which have their absorption peaks in the ultraviolet. The region of maximum light sensitivity is in the near ultraviolet. Since these pigments seem to act as screening pigments, they will be required by the organism as much in visible as in ultraviolet light.

SUMMARY

By the application of spectrophotometric and chromatographic adsorption analysis, the red eye pigment of *Drosophila melanogaster* is shown to consist of a complex of at least eight distinct constituents. Six of these are present in vermilion, cinnabar, and scarlet flies, five in sepia flies.

The overall molecular weight of the red pigment complex is about 300.

A method for the purification and crystallization of one of the constituents is given.

The development of the red pigment complex in cinnabar flies is described in terms of three major components.

Genes of the white allele series are found to affect the amounts of the three major components approximately equally.

ACKNOWLEDGEMENT

The author wishes to thank especially PROF. C. R. DAWSON of the COLUMBIA UNIVERSITY Chemistry Department for providing the facilities of his own laboratory for the chemical part of this work, as well as for many helpful suggestions. He is also indebted to PROFS. L. C. DUNN and TH. DOBZHANSKY and to DR. A. E. MIRSKY for their assistance. He would like to thank MISS REBA MIRSKY for her assistance in this work. Finally he wishes to express his gratitude to PROF. G. WALD of HARVARD UNIVERSITY under whose guidance this study was begun.

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A BIOCHEMICAL BASIS FOR THE PSEUDO-ALLELIC ANTHOCYANIN SERIES IN GOSSYPIUM

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INTRODUCTION

PSEUDO-ALLELISM describes a situation where two members of a supposedly allelic series give a complementary hybrid when crossed together. Typically the parental types are intermediate members of the "series" and the complementary hybrid resembles the top dominant or wild type. The clearest example of this situation is probably the one described by McCLINTOCK (1944) in the case of certain chlorophyll abnormalities in maize. Phenotypically a "series" of four members, normal green, yellow green, pale yellow, and white, were distinguished whose dominance relationships could be expressed as follows:—

(1) Normal green→yellow green→white

(2) Normal green→pale yellow→white

However, the heterozygote, yellow green/pale yellow, gave a complementary hybrid—it was indistinguishable phenotypically from the top dominant, normal green. By a very neat cytological analysis, McCLINTOCK was able to show that the pale yellow and white "alleles" were in reality homozygous deficiencies, the former adjacent to, and the latter including the normal green/yellow green locus. Consequently in the hybrid, pale yellow/yellow green, the deficient section in the "pale yellow" chromosome was covered by the "yellow green" chromosome thus enabling the dominant normal green gene, carried by the "pale yellow" chromosome, to gain full expression.

The successful analysis of the above case in maize gives rise to two points of considerable genetic interest. In the first place it suggests a probable explanation of all complex series of multiple alleles whose members cannot be arranged in a simple quantitative series and in which complementary heterozygotes are found. For example in Gossypium, it provides a reasonable explanation of complementary hybrids found in the "anthocyanin," and "crinkle" series (YU and CHANG, in press; HUTCHINSON 1946). Secondly it may have considerable bearing on such problems as have been recently described by DUNN and CASPARI (1945) in the mouse, where genes having similar though not identical effects are located in nearly adjacent positions on the same chromosome. The origin and significance of such gene complexes is still an open question. Do they arise as duplications (repeats) of an originally single locus? DUNN and CASPARI, in the case of the Brachy-Fused-Kinky complex in the mouse, did not favor this interpretation since they pointed out that the respective loci, though close, were not adjacent, and embryological

studies revealed far greater differences in the development of the three abnormal types than might have been expected from the appearance of the adult animals.

In several respects the anthocyanin complex in Gossypium offers more favorable material for an attack on this problem. The effects on the phenotype are relatively restricted *in character*, and the respective loci cross over with a very low frequency. Furthermore the considerable work previously carried out on the chemistry of the anthocyanin pigments in several plant genera, ROBINSON and ROBINSON (1934 and earlier papers), LAWRENCE and SCOTT-MONCRIEFF (1935), SCOTT-MONCRIEFF (1936), and others, make possible a more detailed comparative study of the action of the genes concerned. The aim of the present investigation was to compare the actions of two genes at adjacent loci on the production of anthocyanin pigment in the flower petal, and to attempt to decide between the two possibilities (1) that the two loci control successive and similar but not identical mechanisms, (2) that they control chemically unrelated steps in a common chain reaction. It is clear that the former possibility would suggest a high degree of homology and therefore favor the hypothesis that the two loci have evolved from an "ancestral" type by duplication.

GENETIC BASIS OF THE ANTHOCYANIN SERIES IN ASIATIC COTTONS

The color of the petal in Asiatic cottons is controlled by two independent genetic systems. One of these determines the color background of the petal, which may be yellow (*Y*), pale (Y^p), or white (*y*) (HUTCHINSON 1931, SILOW 1941). The other (anthocyanin) system determines both the *pattern* in which red pigment is developed and also the *intensity* of that pigment throughout the vegetative and floral parts of the plant. At least twenty distinct genotypes are known which produce a series of phenotypically distinguishable types ranging from a type in which the whole of the vegetative and floral parts is intensely pigmented to a type in which the red pigment is completely absent. All the anthocyanin genes responsible for this series are located in a short section of one chromosome, and until recently were thought to be members of a single multiple allelic series. Actually, in their general breeding behavior, the alternative types do behave as alleles, with the additional feature that their heterozygotes exhibit supplementary phenotypic effects, cf. the *A* series in maize (EMERSON and ANDERSON 1932), the scutes in Drosophila (SEREBROWSKY 1938), color pattern in Harmonia (TAN 1946) etc. Thus a type with red vegetative parts and red margined petal, on crossing with a type having green plant body and red spotted petal will give an F_1 with red plant body and red spotted, red margined petal (SILOW and YU 1942). However, exceptions to true allelic behavior occur, which indicate that more than a single locus is involved. HUTCHINSON (1932) described a complementary action in the case of two "alleles" whose heterozygote resembled phenotypically a third "allele" in the series. The phenotypic appearance of the three types concerned –as they appear when the independent yellow (*Y*) gene is also present—is illustrated on the left of figure 1, (a), (b) and (c). "Red Spot" (a) is by far the commonest

pattern of anthocyanin pigmentation in the cotton flower. In "Ghost Spot" (b) there is no red pigment but a white spot in place of a red spot can be distinguished against the yellow background of the petal. In "Spotless" (c) no spot is present and the petal appears to be a homogeneous yellow. The same relationships hold in white flowered types (*y*) except, of course, that a white "Ghost Spot" on a white background cannot be distinguished phenotypically from "Spotless."

HUTCHINSON found that on crossing Ghost Spot with Spotless the F_1 was phenotypically Red Spot, but was distinguishable by breeding behavior as on selfing it segregated 1 Ghost Spot:2 Red Spot (heterozygous):1 Spotless. In order to explain this complementary interaction HUTCHINSON (1934) adopted a formal interpretation in which the anthocyanin locus was supposed to be divided into two "gene centers" each of which could carry one or more "episomes." It was supposed that in the recessive types episomes would be missing, and for the expression of Red Spot they would be present on both centers. However, the situation is so similar to MCCLINTOCK'S yellow-green/pale yellow interaction in maize, which is known to be the result of pseudo-allelism, that HUTCHINSON'S formal interpretation is no longer necessary. Instead it may be supposed that Red Spot has dominant genes at two adjacent loci, *G S*, Ghost Spot is deficient (or recessive) at one locus, *G O*, Spotless deficient (or recessive) at the other, *O S*, and in addition a fourth type, deficient (or recessive) at both loci, *O O*, is to be expected. This fourth type, "Basic Spotless," has recently been found by YU and CHANG (in press). Phenotypically it is indistinguishable from Spotless (see figure 1d) but, as expected, it behaves as a simple recessive to the other three types, and gives no complementary interaction with Ghost Spot. The four possible combinations of genes (on this basis responsible for the four phenotypes, Red Spot, Ghost Spot, Spotless and Basic Spotless) are illustrated on the right of figure 1.

Critical evidence for the pseudo-allelic nature of the anthocyanin series has recently been provided by YU and CHANG (in press) who have produced convincing evidence that the anthocyanin "locus" consists of a series of sub-units, probably arranged in a definite linear order, and certainly separable by the normal mechanism of crossing over. (The possibility that certain new "mutant" types had arisen by a form of intra-allelic exchange in heterozygotes had previously been considered by SILOW and YU (1942) but at that time critical evidence was lacking.) At least three sub-units (loci), and probably more, are necessary to explain the twenty pseudo-allelic types at present known.

In this paper attention will be confined to the two loci which control presence or absence of petal spot, and its pigmentation when present; that is the four combinations of genes illustrated in figure 1. Solely from the physiological viewpoint there is nothing unusual in a situation where two acyanic flower types give a hybrid with cyanic flowers. Similar cases have been observed in Lathyrus (BATESON, SAUNDERS and PUNNETT 1905), Antirrhinum (WHELDALE 1916), and Cheiranthus (GAIRDNER, cited by SCOTT-MONCRIEFF 1936) among others. These show merely that at least two chemically independent steps are involved in a chain of reactions leading to the expression of red pig-

ment in the flower. In the present case in Gossypium, however, there is the additional problem of why the genes controlling these two steps are located adjacently in the chromosome. One possibility is that the association is a chance one; that the loci are intrinsically unrelated and govern different pro-

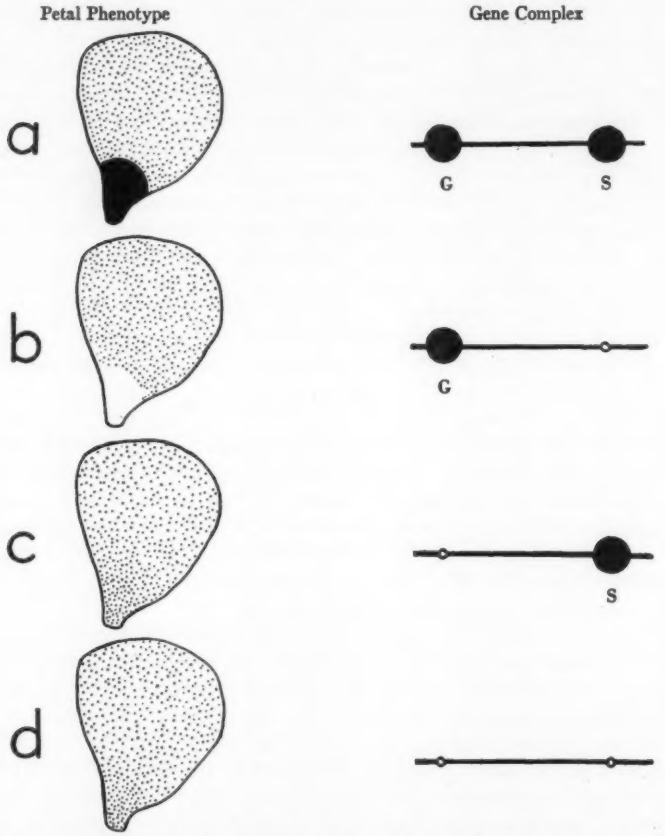


FIGURE 1.—Diagram illustrating the relation between four pseudo-alleles in the anthocyanin series and their effect on the petal phenotype in Asiatic cottons.

cesses which happen to be successive steps necessary for pigment expression. The other possibility is that the loci have arisen by duplication from a single "ancestral" locus, and that associated with (or subsequent to) that duplication, the "daughter" loci have differentiated—the differentiation, of course, is necessary to explain the complementary interaction observed between genes at the two loci. Now if the adjacent position of the loci is a matter of chance, there is clearly no reason to suppose any close relationship between the processes they

govern. If, alternatively, the loci are duplicates, then they must initially have been homologous and have governed homologous chemical processes. The nature of the chemical reactions governed by genes at the two loci should provide valuable evidence in discriminating between the above alternatives.

COMPARATIVE STUDIES OF PIGMENT DEVELOPMENT IN THE FLOWERS OF RED SPOT, GHOST SPOT, SPOTLESS AND BASIC SPOTLESS TYPES

The diagrams in figure 1 illustrate the appearance of the petals in newly opened flowers. Flowers of all Gossypium species fade on the afternoon of the day on which they open. Fading behavior differs in the four flower types under consideration. In Ghost Spot and Basic Spotless flowers, the petals wither and eventually become dry and brown before they are shed. In Red Spot and Spotless flowers, on the contrary, a red flush suffuses the petal at the onset of fading —the formerly acyanic parts become cyanic. Reference to figure 1 shows that the types which produce a red flush on fading, Red Spot and Spotless, both carry a gene at the S locus ,while Ghost Spot and Basic Spotless types do not.

Again referring to figure 1, it can be seen that the spotted types, Red Spot and Ghost Spot, carry a gene at the *G* locus, while the spotless types, Spotless, and Basic Spotless, do not. In formal genetic terms, then, the presence of a spot on the petal is dependent on *G*, and the pigmentation of that spot when present is determined by *S*.

In order to examine the phenotypic differences in more detail, petals of the four types under consideration were examined at successive stages of development, beginning at the time when the petals first protrude through the calyx sheath, that is at approximately the stage when the pollen mother cells are undergoing meiosis. The petals were flattened between two glass slides and examined by reflected light under the low power magnification of a dissection microscope. Blue light was found to be the best source for distinguishing between yellow pigmented and unpigmented tissues. The results of this comparative study are illustrated diagrammatically in figure 2. At the earliest stage the petals in all four types were unpigmented, but at subsequent stages all the cells in surface view became filled with the yellow pigment. The course of development of the yellow pigment was to some extent obscured by the fact that chlorophyll was also present in these early stages, so that the petals appeared yellow-green instead of yellow. The impression was gained, however, that the yellow pigment develops first in the cells towards the outer edges of the young petal, and later the pigmented region extends towards the petal-base. *There was no doubt however that there is a stage, common to all four types of petal examined, in which all the cells visible in surface view contain yellow pigment.*

From this stage onwards, in Basic Spotless and Spotless petals no further changes in pigmentation occurred, except that as growth continued, chlorophyll disappeared from the tissues leaving them bright yellow in color. In Ghost Spot petals from seven to ten days before flowering, the yellow pigment began to disappear from the future spot area of the petal, leaving white

streaks or patches of unpigmented cells. With continued disappearance of the yellow pigment the white patches coalesced, finally producing a homogeneous white spot characteristic of the mature Ghost Spot petal. In Red Spot petals at approximately the same time, a similar disappearance of the yellow pigment in the spot area occurred, but in addition, groups of cells in the resulting white streaks began to develop *red* pigment. These red areas then increased

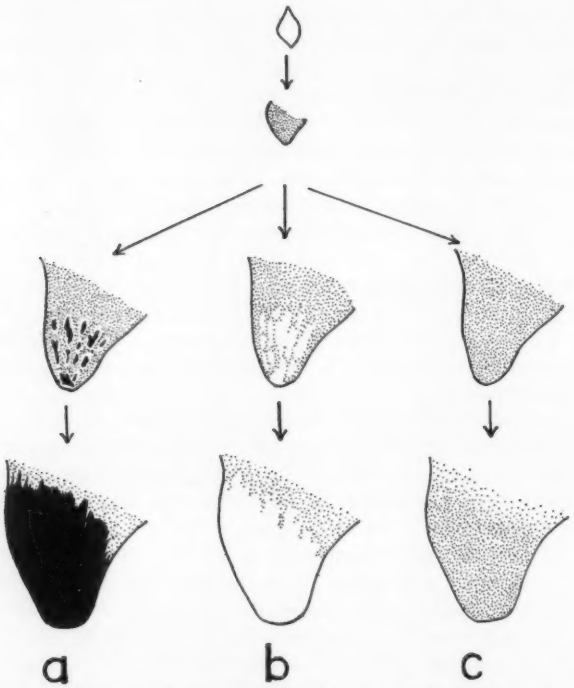


FIGURE 2.—Diagram illustrating distribution of pigments in the "spot area" of the petal at successive stages of development in the four types (a) Red Spot, (b) Ghost Spot, (c) Spotless and Basic Spotless. Anthocyanin indicated by black, leucosubstance by white, and anthoxanthin by stippled areas.

in size as neighboring unpigmented cells developed red pigment, and eventually they, too, coalesced giving rise to the homogeneous red spot of the mature petal. It is clear from these observations that the production of a red spot depends on two successive steps: first the disappearance of the yellow pigment from the cells situated towards the base of the petal, and second, the development of red pigment in these yellow-free cells. The first step is obviously under the control of the gene, *G*, and the second step under the control of the gene, *S*. Furthermore, these observations suggest that red pigment is not developed in cells of the potential spot area until the yellow pigment has disappeared from

them, which furnishes an explanation of the complementary interaction between *G* and *S* in the formation of a red spot.

It will be convenient at this point to anticipate certain results of the chemical findings which will be considered in the following sections. ROBINSON and ROBINSON (1935) and other workers have found that certain plant tissues, including acyanic flower petals, contain colorless (leuco) substances which are readily converted into red (anthocyanin) pigments on boiling with acids. Accordingly, extracts were made from petals of the three acyanic flower types Ghost Spot, Spotless and Basic Spotless. Equal numbers of petals from each type were ground in equivalent volumes of one percent hydrochloric acid. To each filtered extract was added an equal quantity of concentrated hydrochloric acid and the solutions were then boiled for a few minutes. Extracts from Ghost Spot petals developed a deep red color under this treatment, which was later shown to be due to an anthocyanin pigment. By contrast, only faint pink colors were developed by the Spotless and Basic Spotless extracts. Subsequently it was found by extracting the base of the petal, that is the portion roughly corresponding to the spot area in spotted forms, separately, that the bulk of the leuco-substance was present in the spot area.

Having found that Ghost Spot petals differ from Spotless and Basic Spotless petals, not only phenotypically but also quantitatively in respect of their leuco-substance content, it seemed necessary to trace the origin of the latter difference by testing extracts of developing petals at equivalent stages. The tests showed that petals from the youngest buds of the three acyanic, and also of Red Spot flower types, were rich in leuco-substance. At this stage no yellow pigment could be detected, though it may of course have been present in small amounts. Increasingly older flower buds of the three acyanic types continued to give positive tests for leuco-substance until about two days before flowering. At this stage the leuco-substance rapidly disappeared from Spotless and Basic Spotless petals, and at maturity, as described earlier, very little remained. In Ghost Spot petals, on the contrary, a high content of leuco-substance was maintained.

In summary, the results of these observations would appear to justify the following conclusions:—

(1) In Spotless and Basic Spotless, which lack the gene *G*, the petals from the earliest stage onwards contain a leuco-substance. This substance disappears rapidly shortly before flowering. During the same developmental period, a yellow pigment accumulates in all the visible cells of the petal.

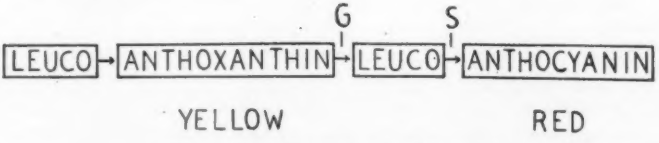
(2) In Ghost Spot, where the gene *G*, is present, the petals also contain a leuco-substance from the earliest stages onwards. This substance does not disappear at maturity, and is particularly abundant in the spot area. During the same developmental period, the yellow pigment disappears from the spot area.

(3) Differences between (1) and (2) above may be ascribed to the action of the gene *G* whose presence is therefore associated both with the disappearance of the yellow pigment from the spot area and also the maintenance of the leuco-substance content in the same area when the petal matures. This apparently

dual role could be interpreted as a single process if one assumption were made, *namely that the gene G converts the yellow pigment to leuco-substance.* In that case the disappearance of the yellow pigment would be due to its conversion into leuco-substance, which in turn would account for the accumulation of the latter in the mature Ghost Spot petal. Independent evidence which suggests that the yellow pigment can be converted into leuco-substance is provided by the fading behavior of Ghost Spot petals. Tests showed that two days after flowering, the faded petals contained very little yellow pigment as compared with fresh petals, but that this fall in yellow pigment content was associated with a rise in quantity of leuco-substance. Similarly, in Red Spot petals, the amount of yellow pigment diminishes during fading, while at the same time, the parts of the petal which are acyanic at flowering, develop a red flush.

(4) Differences between Ghost Spot (*GO*) and Red Spot (*GS*) may be accribed to the effect of the gene *S*. It is known that Ghost Spot contains leuco-substance in the spot area, while Red Spot contains red (anthocyanin) pigment in the same region. Since the leuco-substance can be readily converted into an anthocyanin pigment *in vitro*, since Red Spot contains the gene *G* responsible for the accumulation of leuco-substance in the spot area, and since the developmental evidence shows that a white spot precedes the formation of a red spot in the Red Spot petal, it is reasonable to suppose that *the gene S converts the leuco-substance to an anthocyanin.*

(5) If the interpretations offered in (3) and (4) above are correct, then it seems highly probable that the leuco-substance and the yellow and red pigments may be closely related chemically. Further, since the leuco-substance is present in the petals of all four types at a very early stage—before either the yellow or red pigments can be detected—it is possible that it may be a common precursor of both the yellow and red pigments. In short, the following relationship between the three substances may be proposed *as a working hypothesis* on which to base further tests:—



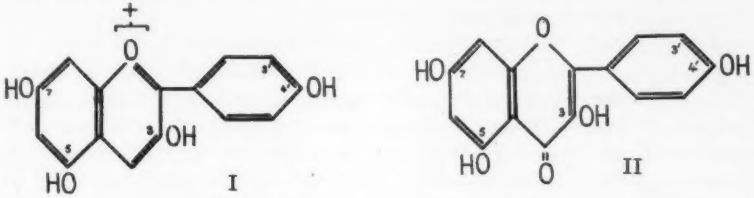
Pigments in the spot area of the petal

The red pigment did not appear until *after* the yellow pigment had appeared and again disappeared from the spot area of the petal. It is reasonable to conclude that Spotless petals which contain both the *S* gene, and, at an early stage, leuco-substance, do not produce red pigment because the yellow pigment has already been formed and the *S* gene does not work in cells containing yellow pigment. In order to test the validity of the hypothesis it was necessary to investigate the chemical nature of the red and yellow pigments, and their interrelations with the leuco-substance. These will be considered in the following section of this paper.

THE CHEMICAL BASIS OF FLOWER PIGMENTATION IN GOSSYPIUM

General

The sap soluble pigments in flowers fall into two main classes (1) the red or purple pigments (anthocyanins) and (2) the yellow pigments (anthoxanthins). Their chemical structures and interrelationships have been reviewed from the genetic point of view by SCOTT-MONCRIEFF (1936) and LAWRENCE and PRICE (1940). The basic structures of the two classes of pigments are closely similar as indicated below:—



The anthoxanthins may be further divided into two sub-groups, the *flavonols* (as in II above) and the *flavones* (in which no substituent hydroxyl group is present at position 3).

The numerous anthocyanin and anthoxanthin pigments which have been isolated, differ in the number and character of substituent groups (chiefly—OH, and —OCH_3) which are present in the lateral benzene ring at positions, 3′ and 5′. Thus the formula of the anthocyanin pigment, *cyanidin*, is derived from the basic structure, I, by substitution with a hydroxyl group at position 3′. The analogous flavonol pigment, *quercetin*, is derived by exactly similar substitution in the basic structure, II. The anthocyanin pigment, *malvidin*, is derived from the basic structure I by substitution with —OCH_3 groups at positions 3′ and 5′ and so on.

In the plant the pigments apparently do not occur in the free state but are combined with various sugars to form *glycosides*—*monoglycosides* when a sugar molecule replaces the hydroxyl at position 3, and *diglycosides* when hydroxyls at both positions 3 and 5 are replaced. In strict terminology, *anthocyanin* refers to the glycosidal form, and when the sugars are removed by hydrolysis the resulting aglycone is known as an *anthocyanidin*. The considerable work which has been carried out on the anthocyanin pigments shows that the substitution with hydroxyl and methyl groups in the nucleus, and the nature of the sugar attachments is under the control of specific genes. Almost certainly the same situation occurs in the case of the anthoxanthin pigments, but less information is available at the present time.

During the early researches in flower pigment chemistry, the similar structures of anthocyanins and anthoxanthins suggested that their syntheses were interrelated (WHELDALE 1916, 1920). When EVEREST showed (1914) that the anthocyanin pigment, cyanidin, could be prepared *in vitro* by strong reduction of its analogous anthoxanthin quercetin, it was thought probable that the anthoxanthins were actually precursors of the anthocyanins during synthesis

of the latter in the plant. In recent years this view has fallen into disfavor, and the view more generally held is that the two classes of pigment are synthesized in parallel from a common precursor (see SCOTT-MONCRIEFF 1936, LAWRENCE and PRICE 1940). One of the chief arguments that has been advanced in support of parallel synthesis, is that if anthocyanins were synthesized sequentially from anthoxanthins, it would be expected that analogous types of the two pigments would be found in the same flower. Actually studies have shown that when anthocyanins and anthoxanthins occur together, they are frequently not analogous but differ in the nature of their substituent groups. From the genetic point of view, however, this argument scarcely constitutes a valid criticism of the alternative, sequential hypothesis (SANDO *et al.* 1922, 1935). Parallel syntheses of these structurally similar pigments would surely require that the genes controlling these syntheses should be extremely specific in their actions. Otherwise a gene responsible for a particular substitution in an anthocyanin molecule might be expected to bring about a similar substitution in the accompanying anthoxanthin. However if this situation is avoided by postulating extreme specificity of the genes, the whole argument in favor of parallel syntheses is much weakened. A situation involving sequential synthesis might be equally probable. For example, a certain genotype might contain specific genes for converting quercetin into cyanidin, and in addition a specific gene for methylating the cyanidin nucleus. In that case one would not necessarily expect quercetin to be accompanied by cyanidin in the same flower, since the cyanidin might easily be converted into a methylated form (that is, peonidin) as fast as it was formed. It seems therefore that a critical discrimination between the alternative hypotheses of parallel or sequential syntheses must await more information on the specificity of the genes concerned.

Rapid methods for the isolation and identification of the anthocyanin pigments have been developed by ROBINSON and ROBINSON (1934 and earlier papers) and have been summarized by SCOTT-MONCRIEFF (1936). As far as the writer is aware no equivalent methods have been developed for anthoxanthins. Both pigments are readily extracted by grinding the petals with dilute hydrochloric acid. The mixture of pigments so obtained can be separated by exhaustive extraction with ethyl acetate, in which the anthoxanthins are soluble, anthocyanins insoluble. The anthocyanins present in the anthoxanthin-free extracts can then be identified by their characteristic color reactions with certain reagents; sodium hydroxide, sodium carbonate, sodium acetate and ferric chloride. Determination of their glycosidal structure can be made by examining the partition of the pigment between two solvents, water and amyl alcohol. More detailed confirmatory studies depend on colorimetric comparison with standard pigments, and on spectrophotometric methods.

The anthoxanthins as a class may be detected by the yellow color produced with alkalies, their yellow or orange-yellow precipitates with lead acetate, and the olive or brownish colorations produced with ferric chloride. Some anthoxanthins can be converted to anthocyanins by reduction with nascent hydrogen. Discrimination between different anthoxanthins, however, depends on purification, determinations of melting point, and spectrophotometric methods.

The anthocyanin pigments in flowers of Gossypium species

As far as the writer is aware no chemical studies of the anthocyanin pigments in Gossypium have been made previously, although they have received considerable attention from the genetic aspect (HARLAND 1929, HUTCHINSON 1932, SILOW 1941, SILOW and YU 1942, YU and CHANG, in press). In the work to be described, the chemistry of the anthocyanin pigments in flowers of the following species was examined.

G. arboreum, *G. herbaceum*—Asiatic diploid species (cultivated)
G. barbadense—New World amphidiploid species (cultivated)
G. klotzschianum—American diploid species (wild)
G. anomalum—African diploid species (wild)

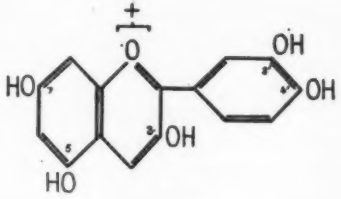
All of these, except *G. anomalum*, were similar phenotypically in having yellow flowers (*Y*), with a red, or more properly a magenta, spot at the base of the petals (as in figure 1a). The flowers of *G. anomalum* contain a pale yellow anthoxanthin pigment. In addition they have a spot at the base of the petal which is purple in color. The purple pigment is not confined to the spot area but is present in small amounts throughout the petal, so that the interaction between pale yellow and purple pigments produces a pale lilac flush on the main body of the petal. On placing the petals of *G. anomalum* in dilute acid, however, the purple color is immediately reddened, and the extract obtained by grinding the petals in one percent hydrochloric acid has the same, magenta, color as the extracts of the petals of the other four species listed above, prepared in the same way.

The crude acid extracts were made anthoxanthin-free by shaking repeatedly with ethyl acetate. The purified solutions of anthocyanin were next subjected to the qualitative color tests listed by SCOTT-MONCRIEFF (1936, p. 121). Identical color reactions were obtained with the anthocyanin extracts from the petals of all five species. They are summarized below:—

Reagent:	Sodium hydroxide	Sodium carbonate	Sodium acetate
Color:	Pure blue	Violet blue	Red-violet

Alcoholic solutions of the anthocyanins were prepared by grinding the petals with ethyl acetate, followed by repeated washing with the same solvent to remove the last traces of anthoxanthin, the anthocyanin pigment then being taken up in ethyl or amyl alcohol. In all cases the alcoholic solution gave a deep blue color on adding a few drops of ferric chloride. This combination of reactions is characteristic of monoglycosides of *cyanidin*. The glycosidal nature of the pigment was confirmed by examining its partition between aqueous and amyl alcohol solvents. In the strengths employed, the pigment was about equally distributed between the two solvents after shaking. On dilution with water, the distribution in the alcoholic layer was greatly decreased, but on saturating with sodium chloride the pigment was almost completely taken up by the amyl alcohol. This reaction is characteristic of pentose glycosides, and forms a ready means of distinguishing them from monoglycosides and diglycosides. It seems probable therefore, in view of the fact that the reactions of the

anthocyanin extracts were identical in the case of all five species examined, that cyanidin-3 pentose glycoside is the commonest, if not the only anthocyanin pigment present in the genus. The hydrolysed form has the following structure:



and in the glycosidal form the hydroxyl at position 3 would be replaced by a pentose molecule.

Subsequent examination of a white flowered (*y*) type of *G. arboreum* with red spotted petals showed that its anthocyanin pigment was also cyanidin 3-pentose glycoside. It may therefore be concluded—since yellow, pale yellow, and white flowers, containing different anthoxanthins, all contain the same anthocyanin pigment—that the genes governing the form of anthoxanthin pigment do not directly affect the form of anthocyanin produced in the petal, that is, they are highly specific.

The anthoxanthin pigments in flowers of Gossypium species

The early work of PERKIN (1899, 1909, 1916), later extended by VIEHOVER, CHERNOFF, and JOHNS (1918) showed that three anthoxanthin pigments occur in the petals of cotton flowers. The distribution of these pigments in the different species is summarized in table 1.

TABLE I

Distribution of three anthoxanthin pigments in five species of Gossypium.

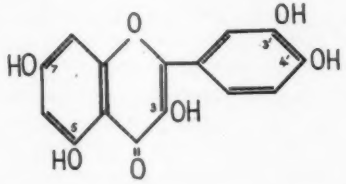
SPECIES	PETAL COLOR	ANTHOXANTHIN CONTENT		
		Gossypitrin	*Quercimeritrin*	*Isoquercitrin*
G. arboreum	Yellow	++	−	+
G. herbaceum†	Yellow	++	−	+
G. arboreum	White*	−	−	−
G. barbadense	Yellow	+	++	+
G. hirsutum	White	−	++	+

* Traces of unidentified anthoxanthin found.

† A fourth anthoxanthin, *herbacitrin*, was later found in this species by NEELAKANTAM and SESHADRI (1937).

Isoquercitrin and *quercimeritrin* are isomeric glucosides (that is, glycosides involving the sugar glucose) of the same anthoxanthin, *quercetin*. They differ only in the position of attachment of the glucose molecule on the quercetin

nucleus. *Gossypitrin* is a glucoside of *gossypetin*. Its empiric formula was found by PERKIN to contain an additional oxygen atom, as compared with that of the other two pigments. Later, BAKER, NODZU, and ROBINSON (1929) showed by synthesis that its structural formula consists of a quercetin nucleus in which an additional hydroxyl (—OH) group has been substituted. Reference to table 1 shows that isoquercitrin is common to all flower types examined, with the possible exception of white flowered types of *G. arboreum*, which contained only traces of anthoxanthin pigment. The amount of pigment obtained in these flowers, PERKIN found, was insufficient to carry out critical chemical analysis with the methods employed. With this reservation however, the evidence indicates that quercetin is the primary anthoxanthin to be synthesized in the flower. It has the following structural formula:—



The yellow flowered types (genetically, *Y*) differ from the others in the fact that they contain gossypitrin, which is also their principal pigment. It is clear that *Y* is associated with the presence of gossypitrin, and its probable genetic action is the conversion of quercetin to gossypetin by hydroxylation.

A further point of interest is that New World cotton flowers contain quercimeritrin, which is absent from the flowers of the Asiatic species which have been studied chemically. In current genetic terminology, both white flowered New World and white flowered Asiatic types are symbolized *y*, which is obviously inconsistent with the chemical evidence. Neither is it consistent with the fact that hybrids between white flowered New World cottons and white flowered Asiatic cottons have *yellow* flowers. However, SILOW (1941) has shown that a certain pale yellow flowered type of *G. arboreum* known as "Chinese pale," and the pale yellow flowered *G. anomalum*, also give complementary yellow flowered hybrids on crossing with standard pale yellow (Y^p) and white (*y*) flowered types of *G. arboreum*. It is clear that the *Y* series and its 'duplicates" need reinvestigation on a chemical basis.

In the present study, further investigations of a preliminary nature were carried out on two flower types in which chemical evidence is still lacking, namely *G. anomalum* (pale yellow flower) and *G. arboreum* (white flower, *y*). The anthoxanthin pigments were extracted with ethyl acetate, filtered, evaporated to dryness and taken up in alcohol. The alcoholic solutions were pale yellowish-green in color. Routine tests were then carried out on both extracts, using as a control a similarly prepared extract of white flowered *G. hirsutum*, ("*y*"), in which the anthoxanthin pigment is known to consist principally of quercimeritrin (see table 1). The results of the tests are summarized below:—

Reagent:—	Basic lead acetate	Sodium carbonate	Ferric chloride	Strong hydrochloric	Nascent hydrogen
G. hirsutum("*y*")	Orange ppc.	Golden yellow	Olive green	Golden yellow	Magenta
G. anomalum	Orange ppc.	Golden yellow	Olive green	Golden yellow	Magenta
G. arboreum(*y*)	Lemon yellow ppc.	Lemon yellow	Pale olive	Yellow-green	Magenta

No difference was apparent between the anthoxanthin pigments of the *G. hirsutum* and *G. anomalum* flowers. On evaporating their solutions to dryness and recrystallizing from hot water, both were found to deposit mixed crystals of plate and needle-like forms. According to PERKIN quercimeritrin differs from isoquercitrin and gossypitrin in the fact that it crystallizes in glistening plates; the other two pigments have needle-like crystals. It seems highly probable therefore that the petals of *G. anomalum*, like those of *G. hirsutum*, contain quercimeritrin. Similarity in form of anthoxanthin pigment, in addition to the previous genetic evidence that both species give yellow flowered hybrids on crossing with white flowered *G. arboreum*, suggests that both contain the same genotype in respect of anthoxanthin pigmentation and that therefore the present symbol, *y*, for *G. hirsutum* is incorrect.

The quantity of anthoxanthin pigment present in the white flowered *G. arboreum* petals was much less than in the *G. hirsutum* and *G. anomalum* petals. Furthermore, the tests tabulated above show that a qualitative as well as a quantitative difference is involved.

The leuco-substance in Ghost Spot flowers of Gossypium

Evidence for the presence of a leuco-substance

Since anthocyanins and anthoxanthins give blue and yellow colorations respectively in alkaline solution it is to be expected that crude extracts of petals in which both pigments occur will give a green color on treatment with alkalies. This was found to be the case in all the cyanic (Red Spot) flowers tested. Crude extracts of acyanic flowers of the Spotless and Basic Spotless types, since they contained no anthocyanin gave yellow colors with alkalies. Crude extracts of both yellow flowered (*Y*) and white flowered (*y*) Ghost Spot petals of *G. arboreum*, however, gave unexpected results. Although the petals of Ghost Spot flowers are acyanic, and although the crude extracts were pale yellow or colorless in acid solution, yet, on treating with alkalies the extracts usually gave a transient *green* color which changed into the expected yellow color on standing.

If the green solution was immediately made acid again with a few drops of hydrochloric acid, the green color was converted to pink. In short, the crude extracts of Ghost Spot petals, after being made alkaline, behaved as if traces of anthocyanin were present.

When the crude extracts in dilute acid solution were rendered free from anthoxanthin by repeated shaking with ethyl acetate, it was found that they no longer gave any color reaction with dilute alkalies, though tests of the ethyl acetate washings showed that no "anthocyanin-like" substance had been removed. On adding a few drops of concentrated hydrochloric acid to the anthoxanthin-free extracts and then boiling, a magenta color was rapidly developed. The solution was concentrated, and the precipitate separating out was removed by filtration, washed, and taken up in alcohol. On adding a few drops of ferric chloride, a deep blue color was developed, which suggested that the synthesized pigment might be cyanidin, and hence the same as the pigment occurring naturally in cyanic flowers. These results would seem to indicate that large quantities of a leuco-substance are present in Ghost Spot petals, that this substance, like the anthocyanidins, is soluble in alcohol and insoluble in ethyl acetate, and is rather easily converted into a true anthocyanidin pigment.

The nature of the conversion of leuco-substance to anthocyanidin

The ROBINSONS (1935) considered that the production of the anthocyanidin, peltogynidin, from its leuco-form, peltogynol, by boiling with hydrochloric acid probably depended on an oxidation process, although attempts to exclude oxygen during the conversion did not inhibit the reaction. In the present case, independent lines of evidence have been obtained which show that the leuco-substance in Gossypium petals on the contrary, is reduced (hydrogenated) during its conversion to an anthocyanidin. Preliminary experiments showed that while anthocyanin solutions were readily decolorized on shaking with hydrogen peroxide in acid solution, they were remarkably stable in the presence of reducing agents. The color was not discharged on boiling with formalin, and only with difficulty when the boiling solution was treated with nascent hydrogen. Finally two methods were found which made it possible to bring about a reversible reaction, leuco-substance⇄anthocyanidin readily *in vitro*. It is well known that methylene blue may act as a hydrogen acceptor in certain reactions, being reduced to a colorless form in the process:—

$$\underset{\text{(blue)}}{MeB} + 2H \longrightarrow \underset{\text{(colorless)}}{MeBH_2}$$

Methylene blue is stable on boiling with hydrochloric acid, and the color is only slowly discharged (presumably due to disintegration of the methylene blue nucleus) when hydrogen peroxide is added to the boiling acidic solution. The leuco-substance extracted from Ghost Spot petals is converted into an anthocyanidin on boiling with hydrochloric acid, and the color of the latter is immediately discharged on adding hydrogen peroxide. It was found that when methylene blue was boiled with the leuco-substance in dilute acid solution, no color change took place, but on the addition of a few drops of hydrochloric acid to the boiling solution the color was rapidly discharged. As the last trace of the blue color disappeared, the magenta anthocyanidin color began to develop. On adding a few more drops of methylene blue the magenta color was discharged, but again appeared on continued boiling. In this manner the

reversible color change, blue⇄magenta could be continued indefinitely until the acid medium became very dilute, at which stage the blue color remained and no further anthocyanidin was produced. The reactions involved may be represented as follows:

$$\underset{\text{leuco-substance}}{A} \xrightarrow{+HCl} \underset{\text{anthocyanidin}}{AH} \qquad (1)$$

The anthocyanidin yields hydrogen to the methylene blue until all the latter is converted into its colorless (reduced) form:

$$2AH + MeB \longrightarrow 2A + MeBH_2 \qquad (2)$$

at which stage equation (1) goes to completion. That the decolorization of the methylene blue is due to its accepting hydrogen from the anthocyanidin is confirmed by the fact that i. hydrogen peroxide is added to the system when the anthocyanidin is present, the magenta color is immediately discharged, and simultaneously the methylene blue color is restored.

Additional evidence of the nature of the reaction was obtained by mixing anthocyanin extracts from Red Spot petals with methylene blue. When both original solutions were concentrated, a deep violet color due to a mixture of the blue and magenta pigments was produced. With more dilute solutions however it was noticed that there was a considerable reduction in the densities of the pigments after mixing. It seems possible that at this stage a partial dissociation had taken place, giving an equilibrium mixture of the four components:—

$$MeB + 2AH \rightleftarrows MeBH_2 + 2A$$

On boiling with strong hydrochloric acid the magenta anthocyanidin color was rapidly recovered, and the methylene blue was consequently all converted into its colorless form as shown by equations (1) and (2) above. Their similarity in reaction with methylene blue provides additional evidence that the natural and synthesized anthocyanidins are structurally very similar.

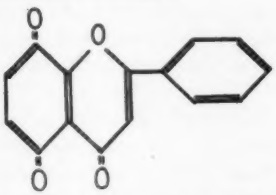
A parallel reaction was obtained by warming a three percent hydrochloric acid solution of anthocyanin from Red Spot petals, with a mild oxidizing agent, potassium ferricyanide. To the solution containing both substances, an equal volume of amyl alcohol was added. The mixture was shaken and then allowed to settle out into two layers; the upper, alcoholic layer (pink) containing the anthocyanin, and the lower, aqueous layer (yellow) containing the potassium ferricyanide. On warming, with frequent shaking, the potassium ferricyanide was slowly reduced as shown by the production of a fine blue suspension of the characteristic ferro-ferricyanide salt at the interface, and a change from yellow to green color in the lower layer. At the same time the pink color of the upper layer disappeared. Apparently the potassium ferricyanide had oxidized the anthocyanin to a colorless leuco-substance. This was confirmed by reversing the reaction. A few particles of granulated zinc were dropped into the solution and the nascent hydrogen thus generated slowly completed the reduction of the unchanged ferricyanide and ferro-ferricyanide remaining in the lower

layer, and at the same time restored the pink color of the upper layer. This reaction and the reaction with methylene blue leave no room for doubt that, the change from leuco-substance to anthocyanin *in vitro* involves chemical reduction. (Since the above was written, it has been found that anthoxanthin-free alcoholic solutions of leuco-substance extracted from Ghost Spot petals can be reduced directly to anthocyanin with nascent hydrogen. The reaction does not take place in aqueous solution.)

The relation between leuco-substance and anthoxanthin

A one percent hydrochloric acid solution of the leuco-substance, from which the anthoxanthin had previously been separated with ethyl acetate was boiled with a few drops of one percent hydrogen peroxide. A yellow color developed in the solution. With excess hydrogen peroxide a dull yellowish brown suspension also appeared and separated out on standing. The dull yellowish brown precipitate was easily removed by filtration and the clear pale-yellow filtrate was found to possess some of the general properties of an anthoxanthin, namely it was soluble in ethyl acetate, gave a golden yellow color with alkalies and a pale yellow precipitate with basic lead acetate.

It seems probable that the dull yellowish brown precipitate also obtained on oxidation was a further oxidation product, since PERKIN (1916) found that the anthoxanthins could be readily oxidized to corresponding quinones which have the following basic skeletal structure:—



Although there can be no critical information on this point without detailed chemical analysis, two lines of evidence make it probable. In the first place, similar dull yellowish brown precipitates settle out when acid solutions of natural anthoxanthins from Gossypium petals are boiled with hydrogen peroxide, which suggests that the anthoxanthin and leuco-substance can be oxidized to similar substances. Secondly, PERKIN found that the anthoxanthins can be oxidized, either in the hydrolyzed or glycosidal states, to the corresponding quinones. Thus the hydrolyzed anthoxanthin pigment, gossypetin, could be oxidized to the quinone, *gossypitone*, and similarly in the corresponding glycosidal state, gossypitrin could be oxidized to *gossypitrone*. Gossypitrone is red in color and can be converted to its corresponding hydrolyzed form, gossypitone, which has a pale brown color. In the present studies it was frequently found that oxidation of the anthoxanthins from yellow petalled flowers (containing gossypitrin) gave a transient red color (gossypitrone?) which rapidly changed to the dull yellowish brown color (gossypitone?) due presumably to hydrolysis in acid solution.

It is clear that if it could be shown that the *in vitro* conversion of quercetin

to cyanidin was accomplished *via* an intermediate leuco-substance, then the analogy with the naturally occurring plant pigments would be complete, and no reasonable doubt could be entertained that the change, anthoxanthin→ leuco-substance→anthocyanin actually occurs in the plant. In order to investigate this point, anthoxanthin solutions from Basic Spotless petals (containing gossypitrin and isoquercitrin) were reduced with (1) zinc and *dilute* (one percent) hydrochloric acid and (2) sulfur dioxide.

(1) The anthoxanthin solution in one percent hydrochloric acid was stirred with a little granulated zinc until the yellow color faded to a pale straw color, at which stage the reaction was stopped, the unconverted zinc being removed by filtration. The unconverted anthoxanthin was next removed by exhaustive extraction with ethyl acetate. The remaining colorless solution was then boiled with strong hydrochloric acid, and a magenta color rapidly developed showing that a leuco-substance had been produced by reduction of the anthoxanthin, and subsequently converted to an anthocyanin in strong acid solution.

(2) A similar anthoxanthin solution to that used in (1) above was boiled with excess sodium bisulfite until sulfur dioxide was no longer evolved. The sodium chloride and unchanged sodium bisulfite was removed by adding a larger bulk of 95 percent alcohol and filtering; adding a few drops of dilute hydrochloric acid to the filtrate and filtering again. The final filtrate had a clear pale yellow color. It was diluted to twice its bulk with water and then concentrated to its original volume, in order to remove most of the alcohol. On shaking with ethyl acetate, the yellow color was removed, showing that it was due to unconverted anthoxanthin. The remaining almost colorless solution yielded large quantities of anthocyanin on boiling with strong hydrochloric acid.

Although the above experiments only refer to the anthoxanthin solutions obtained from Basic Spotless petals, quite parallel reactions were obtained with the anthoxanthins from petals of *G. anomalum* and white flowered *G. arboreum*, the only observable difference being that in the case of the last two types very little unconverted anthoxanthin remained in the solution after reduction. It is not known whether this difference is attributable to the qualitative differences between the anthoxanthins concerned, or merely to quantitative differences in anthoxanthin content of the respective solutions. However since the anthocyanin pigment obtained in each case appeared to be cyanidin, since it gave a blue coloration with ferric chloride in alcoholic solution, it is likely that any gossypitrin present in the original anthoxanthin solutions was first reduced to quercetin, and subsequently converted to cyanidin *via* the leuco-substance. The main point is that the leuco-substance represents an intermediate stage in the reduction of an anthoxanthin to an anthocyanin—a situation which is in complete accordance with the genetic evidence and evidence from studies of pigment development presented earlier in this paper.

DISCUSSION

The last two steps in the production of a red spot at the base of the petal in flowers of Asiatic cottons are (1) the replacement of a yellow pigment (anthox-

anthin) by a leuco-substance (2) the replacement of the leuco-substance by a red pigment (anthocyanin) in the spot area. Genetic evidence has shown that (1) and (2) are controlled by the *G* and *S* genes respectively, which are located in adjacent positions on the chromosome. Comparative morphological studies of the interrelations of the three pigment substances concerned, during the development of the petal, suggested that the changes observed might be due to the actual conversion of the anthoxanthin to the anthocyanin pigment in two successive steps, the leuco-substance being an intermediate stage in the conversion. The chemical evidence shows that the primary anthoxanthin pigment in the petal is quercetin, and the corresponding anthocyanidin pigment, cyanidin. Therefore, since in the petal the change, quercetin→leuco-substance→cyanidin, is associated with two simple successive genetic steps, while a similar change can be performed *in vitro* by two simple successive chemical steps, it is a reasonable conclusion that the *in vitro* changes correspond to the gene controlled processes in the petal.

The chemical evidence is consistent with the hypothetical relationship between anthoxanthin, leuco-substance and anthocyanin which was deduced from the genetic and developmental evidence and summarized diagrammatically on page 198. Critical confirmation, however, is required to show that the leuco-substance present in the young buds of all cotton flowers is identical with the leuco-substance present in mature Ghost Spot petals and also with the intermediate colorless substance which can be obtained by reduction of quercetin in aqueous solution *in vitro*. Recently it has been found possible to demonstrate this identity by spectrophotometric methods and the results will be published in detail elsewhere. It would seem, therefore, that the leuco-substance is a common precursor of both anthoxanthin and anthocyanin pigments and chemically intermediate between the two. In the absence of the gene *G* it is oxidized to an anthoxanthin pigment; when *G* is present the anthoxanthin in the spot area is reconverted at a later stage to leuco-substance, and when *S* is also present the latter is further reduced to an anthocyanin pigment. Why Spotless petals, which lack *G* but carry *S* should not produce anthocyanin is difficult to understand, since in the bud stage they, in common with the flowers of the other genetic types, contain the leuco-substance. However, petals of Spotless types, unlike those of the phenotypically identical Basic Spotless types, do produce anthocyanin on fading. Furthermore, Spotless types are known which have considerable quantities of anthocyanin in the petal margins. In such "Red Margin Spotless" types the anthocyanin (but no anthoxanthin) appears at a much earlier stage in the petal margins of the young flower buds that is approximately the same time as the anthoxanthin (but no anthocyanin) appears in the main body of the petals. Presumably the marginal anthocyanin is produced directly from the leuco-substance and does not result from the extended sequence: leuco-substance→anthoxanthin→leuco-substance→anthocyanin which occurs in the spot area of Red Spot types. In short, it appears that there must be some form of competition for the common precursor which inhibits the simultaneous production of anthoxanthin and anthocyanin pigment in the spot area of the petal.

The main purpose of this paper has been to attempt to decide between the two possibilities (1) that the adjacent *G* and *S* loci control successive and similar, but not identical, mechanisms (2) that they control successive but chemically unrelated steps in a common chain reaction. It is clear that the first alternative is the more likely, since the genes control chemically similar reactions, involving chemical reduction of structurally very similar though not identical substrates. *This would suggest that they are daughter genes originating from a common ancestral gene by duplication, and that in the process they have developed specificity.* Such an interpretation requires no new concept, since studies of salivary chromosomes in Drosophila and Sciara (BRIDGES 1936; METZ 1937, 1938; and LEWIS 1945), have demonstrated the existence of serial duplications ("repeats"), and in two cases, Bar and Star/Asteroid, have shown that the cytological repeat is associated with a change in phenotypic expression (a position effect). Other pseudo-allelic series, e.g. scute in Drosophila (SEREBROWSKY 1938, RAFFEL and MULLER 1940) and the pale yellow/yellow-green series in maize (MCCLINTOCK 1944) may have had a similar origin. Evidence from salivary chromosomes does not suggest that extensive serial duplication is common, though this would be necessary to explain the more complex pseudo-allelic series. On the other hand, MCCLINTOCK (1941) has shown that by a specialized mechanism in maize, long series of duplications may sometimes be accumulated. In cotton it may be significant that most of the anthocyanin "mutants" have been obtained in closely related stocks of *G. arboreum*, while in other species fewer variants are known. Possibly in these stocks a single repeat followed by unequal crossing over with the original unmodified chromosome may have given rise to a further duplication (cf Double Bar in Drosophila) and so have initiated a cumulative process. As a result it is conceivable that a series of duplications could have been established as readily as those obtained by MCCLINTOCK with a totally different mechanism.

If serial duplication were a universal mechanism underlying the development of pseudo-allelic systems (which is not proven), it would be difficult to detect it in all but the most favorable material. It would be necessary to show (1) that more than one locus were involved (2) that the adjacent loci had a basic similarity. Cytological demonstration of the association of a supposedly "allelic" series with repeats, or, alternatively, genetic demonstration of rare crossovers within a series whose members controlled similar but not identical processes, would seem to satisfy both conditions. But the former demonstration can only as yet be provided with the use of salivary chromosomes, and the latter requires the identification of specific gene-controlled chemical reactions. However, the existence of complementary interaction between members of a series, with or without supporting cytological evidence, is perhaps indicative of a similar underlying mechanism. For example the *A* series in maize (EMERSON and ANDERSON 1932), has two features in common with the anthocyanin series in Gossypium. The same pigments, quercetin and cyanidin, are involved (SANDO *et al.* 1922, 1935) and the *A* and a^p "alleles" give a complementary heterozygote phenotypically resembling a third "allele," A^b. On the other hand, the *A* series in maize differs from the analogous case in Gossypium in that the basic

recessive "allele," *a*, cannot be fitted readily into a "two loci" interpretation, and no evidence of crossing over has yet been reported. Similarly in a second (*R*) anthocyanin series in maize, the hybrid $R^{g}r^{r}$ phenotypically resembles homozygous $R^{r}R^{r}$ which at first sight would suggest by analogy with cotton that R^{r} is bipartite ($R^{g}r^{r}$). However STADLER (1946) has shown that homozygous R^{r} mutates spontaneously to R^{g} and r^{r} which apparently rules out equal crossing over as a source of these mutants. Nevertheless this evidence does not negate the possibility that the *R* locus is bipartite, or that crossing over may provide an additional source of "mutation." It would seem that observation of such mutants in stocks heterozygous for R^{r} and for other markers in the same chromosome might provide a more stringent test. In the case of the Brachy series in mice, (DUNN and CASPARI 1945), the opposite difficulty is encountered, that is evidence of crossing over has been established but the nature of the material prohibits comparison of gene action in the neighboring loci at a sufficiently critical level. Provisionally it may be permissible to regard all these cases as representing a graded series of the same underlying phenomenon, ranging from very small repeats between which the chances of crossing over are infinitely small to large and complex repeats in which the basic similarity between the duplicates has been lost. This would be consistent with the cytological observations of RAFFEL and MULLER (1940) in the scute series of Drosophila that a quantitative series exists between cytologically undetectable changes (point mutations?) and relatively gross changes in pattern of the salivary chromosomes.

Of particular interest in the present case is the suggestion that repetition of an original ancestral locus has been accompanied by divergence in the functions of the daughter loci. Further the change involved would seem to fulfil the primary requisite for the initiation of *new* loci, and to differ radically from the change involved in a point mutation. Multiple substitution of genes by their mutant alleles should lead to no loss in cytological homology, which would also imply that genes and their mutants, in spite of considerable differences in phenotypic expression, retain considerable biochemical similarity. Otherwise one would have to admit the possibility of complementary interaction between a gene and its mutant allele when combined in a heterozygote, whereas in fact, dominance or intermediacy is the rule. When it has been possible to compare the actions of genes and their mutants at the biochemical level, as in the numerous cases reported in Neurospora, it appears that the change from normal to mutant allele involves a loss or inactivation of the power to control a specific chemical reaction. In contrast to this situation, the production of pseudo-alleles by duplication implies a loss of cytological homology *per se*, and, in the present case at least, a loss of biochemical homology as well. The complementary interaction observed when pseudo-alleles are combined in a heterozygote would be expected to result from such a loss in biochemical homology. Finally, a mechanism leading to divergence in genic function would seem capable of providing more significant variability in the evolutionary sense, than a mechanism in which the major proportion of the variability is attributable to specific inactivations or losses.

It must be admitted that these speculations offer no clue as to the mechanism by which serial duplication *per se* could result in differentiation of the daughter loci at a biochemical level. Although a repeat necessarily involves a local change in chromosomal pattern which could presumably modify genic expression, it would be expected that this "position effect" would be removed when the loci were separated by crossing over. This is apparently only found in the Drosophila cases which have been considered in this paper. Thus in the present case there is no evidence of any phenotypic difference between *G S/O O*, and *G O/O S* types, and similarly in maize the hybrid, yellow-green/pale yellow, is phenotypically green like the homozygous normal type.

SUMMARY

1. The pseudo-allelic anthocyanin series in Asiatic cottons depends on the interaction of at least three neighboring loci two of which (*G* and *S*) govern the presence or absence of a spot at the base of the petal, and its pigmentation when present. When both *G* and *S* genes are present, there is a red spot at the base of the petal; when *G* alone is present the spot is white; and when *G* is absent the petal is spotless.

2. Studies of pigment development in the petal favor the hypothesis that *G* converts a yellow pigment to a leuco-substance, and that *S* further converts the leuco-substance to a red pigment.

3. Chemical studies of the flower pigments show that the primary yellow pigment in the flower is the anthoxanthin, quercetin, and the primary red pigment is the structurally analogous anthocyanidin, cyanidin. The leuco-substance can be converted *in vitro* by *reduction* to cyanidin, and furthermore the naturally occurring anthoxanthin can be reduced *in vitro* to cyanidin via an intermediate leuco-substance.

4. Genetic and chemical evidence combined therefore suggest that the leuco-substance is an intermediate stage and probable common precursor in the natural syntheses of both anthoxanthin and anthocyanin in the petal.

5. If this interpretation is correct the following situation is indicated:—Two genes, *G* and *S*, situated in neighboring loci control similar (reduction) processes and act on very similar though not identical substrates (leuco-substance and anthoxanthin). This would suggest that the loci arose by duplication from a common ancestral locus, and that in the process of duplication, or as a direct result of it, specificity was developed, that is the daughter loci lost both their cytological and biochemical homology and became qualitatively *new* loci.

6. The possible evolutionary significance of these studies is briefly discussed.

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NEGATIVE HETEROSIS AND DECREASED EFFECTIVENESS OF ALLELES IN HETEROZYGOTES[1]

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INTEREST in the causes of heterosis has been renewed again with emphasis on the possibility of its being due to heterozygosity of single loci. Accordingly, any cases of heterozygotes which show effects outside of the range of phenotypes of the homozygotes deserve attention.

One series of such cases was reported recently (STERN, MACKNIGHT, and KODANI 1946). It concerns the cubitus interruptus locus in *Drosophila melanogaster*. For this locus DUBININ and SIDOROV (1934) had shown that the normal allele ci^+ which is dominant over the mutant allele *ci*, looses its dominance in many instances when a chromosome rearrangement has occured in its neighborhood. In consequence, heterozygotes of the constitution $R(+)/ci$, where $R(+)$ designates a "position allele" caused by a rearrangement near the ci^+ allele, possess a phenotype of interrupted veins. Different rearrangements produce genetically different $R(+)$ alleles which may be distinguished by the various mean degrees of vein interruptions produced in the heterozygotes $R(+)/ci$. Out of a series of 17 rearrangements labeled in order of discovery from $R^1(+)$ to $R^{21}(+)$ (of these 21 rearrangements several were not suitable for analysis) four—$R^2(+)$, $R^3(+)$, $R^{12}(+)$, and $R^{15}(+)$—were found to exhibit remarkable properties.

The simplest case is that of $R^{15}(+)$ which may be summarized as follows:

homozygotes $R^{15}(+)/R^{15}(+)$—normal
homozygotes ci/ci—moderate degree of deficient venation
heterozygotes $R^{15}(+)/ci$—extreme degree of deficient venation

Thus these heterozygotes show a "negative heterosis," being more deficient in venation than either of the homozygotes of their component alleles.

For $R^2(+)$ the facts are:

both homozygotes $R^2(+)/R^2(+)$ and ci/ci—moderate degree of deficient venation

heterozygotes $R^2(+)/ci$—extreme degree of deficient venation

Like $R^{15}(+)/ci$, the heterozygotes $R^2(+)/ci$ show negative heterosis.

Similar extreme interruptions of veins are found in $R^3(+)/ci$ and $R^{12}(+)/ci$ heterozygotes but no direct comparison with the translocation homozygotes is possible since $R^3(+)/R^3(+)$ and $R^{12}(+)/R^{12}(+)$ are inviable.

The extreme phenotypes of the four kinds of heterozygotes are significant in

[1] Supported in part by a grant from the Rockefeller Foundation.

a further respect. Hemizygotes for each of these position alleles proved to possess *normal* phenotype. The fact that the "addition" of the recessive allele *ci* to the hemizygous $R(+)$, thus resulting in the heterozygote $R(+)/ci$ causes *deficient* venation may be described as a decrease in the effectiveness of the genic action of the $R(+)$ allele due to the presence of the *ci* allele. Considered in this way, it had remained undecided whether this decrease goes in one direction only—effect of $R(+)$ influenced by *ci*—or whether it is reciprocal, involving the effects of both $R(+)$ and *ci*. If the latter were true the effect of the heterozygote might conceivably be found to be lower, and resulting in more deficient venation than the effects of either one of the constituent hemizygous alleles $R(+)$ and *ci*.

This problem has been attacked on a more favorable material, by using the same $R(+)$ alleles referred to above and, instead of the mutant *ci* allele, the normal iso-allele $+^3$ (STERN and SCHAEFER 1943b). This allele, like the more frequently used normal allele $+^C$ as found in the Canton-S stock, causes wild type venation (at 26°C) both in homozygous or hemizygous constitution. Unlike $+^C$, heterozygotes of $+^3$ with *ci* give a large proportion of individuals with interrupted veins. The allele $+^3$ promised to be a sensitive tool for the discovery of *ci*-like effects of $R(+)$ alleles.

The experiment consisted of crosses between $R(+)$ and $+^3$ in order to study the heterozygotes $R(+)/+^3$. The normal allele involved in the rearrangements was $+^C$ of the Canton-S stock. As a control a cross was made between $+^C/+^C$ and $+^3/+^3$. It yielded 230 $+^C/+^3$ heterozygotes, all normal. *In contrast the heterozygotes* $R(+)/+^3$ *showed varying degrees of deficient venation* (table 1, lines 1–4). For technical reasons the data consist of the F_1 of crosses $R(+)/ey^2 \times +^3$. Two different genotypes, apart from duplication-deficiency types which survive in certain cases, are represented in the F_1, namely $R(+)/+^3$ and $+^2ey^2/+^3ey^+$ ($+^2$ is another wild type iso-allele of *ci*). The latter constitution results in normal phenotypes, as tested in a control cross involving 800 individuals, so that all individuals with deficient venation plus an unknown number of normal overlaps constitute the $R(+)/+^3$ heterozygotes.

In order to relate these results to the problem of heterosis it becomes necessary to distinguish the classical "heterosis by comparison with the homozygotes" from the rarely studied "heterosis by comparison with the hemizygotes." Since homozygotes for $R^{15}(+)$ as well as homozygotes for $+^3$ are normal, while the heterozygotes $R^{15}(+)/+^3$ are deficient in venation, these latter constitute a case of negative heterosis by comparison with the homozygotes, due to heterozygosity at the *ci* locus. In addition, the deficient $R^{15}(+)/+^3$ also represent a case of negative heterosis by comparison with the hemizygotes since both constituent hemizygotes, $R^{15}(+)$ and $+^3$, are normal, or nearly so. Negative heterosis by comparison with the hemizygotes is likewise shown by the venation-deficient heterozygotes, $R^2(+)/+^3$, $R^3(+)/+^3$, and $R^{12}(+)/+^3$, since again all constituent hemizygotes are normal or nearly so. However, $R^2(+)/+^3$ does not represent a case of negative heterosis by comparison with the homozygotes since $R^2(+)/R^2(+)$ are more deficient in venation than the heterozygotes.

The experiments were broadened by obtaining heterozygotes for $+^3$ and four other $R(+)$ alleles, $R^{11}(+)$, $R^5(+)$, $R^8(+)$, and $R^{21}(+)$ (table 1, lines 5–8). These alleles in heterozygotes with *ci* cause only lower mean degrees of deficient venation, within the range of normal and that produced by *ci/ci*. Parallel to the slighter deficient effect of these $R(+)/ci$ heterozygotes as compared with the earlier discussed greatly abnormal $R(+)/ci$ heterozygotes, the heterozygotes $R^{11}(+)/+^3$, $R^5(+)/+^3$, $R^8(+)/+^3$, and $R^{21}(+)/+^3$ as a group cause less deficient venation than the earlier named group of $R(+)/+^3$. Indeed, $R^{11}(+)/+^3$ gave all but one individual which were completely normal, in agreement with the fact that the $R^{11}(+)/ci$ heterozygotes also were less deficient than any other of the 19 $R(+)/ci$ constitutions studied. There exist exceptions to the parallelism in seriation of $R(+)/+^3$ and $R(+)/ci$ phenotypes. They are partly to be expected from secondary genetic complications (see the discussion of $R^3(+)$ and $R^{15}(+)$ in STERN, MACKNIGHT, and KODANI 1946), and partly

TABLE 1

F_1 of 2 $R(+)/ey^2 \times 1+^3/+^3$ 26°C

(Class 0 signifies normal venation, classes 1–4 increasing degrees of deficient venation, STERN, MACKNIGHT, and KODANI 1946)

		♀♀					♂♂				
$R(+)$	Allele	0	1	2	3	4	0	1	2	3	4
$R^{12}(+)$		311	11	56	43	12	258	5	47	59	14
$R^3(+)$		307	8	44	72	7	290	7	35	77	6
$R^{15}(+)$		219	42	56	21	—	212	25	68	31	2
$R^2(+)$		225	27	59	22	1	177	19	60	26	—
$R^{11}(+)$		269	1	—	—	—	295	—	—	—	—
$R^5(+)$		231	31	86	33	—	250	22	67	14	—
$R^8(+)$		174	28	67	43	3	157	28	54	27	3
$R^{21}(+)$		524	85	127	39	1	508	69	132	60	1

due to statistical variations. For the purpose of the present report the three genotypes $R^5(+)/+^3$, $R^8(+)/+^3$, and $R^{21}(+)/+^3$ add further cases in which the heterozygotes between $R(+)$ alleles and $+^3$ show deficient venation. Since the hemizygotes of the alleles $R^3(+)$, $R^{12}(+)$, $R^{15}(+)$, and $R^2(+)$ as well as the hemizygotes of three other $R(+)$ alleles studied by DUBININ and SIDOROV are normal in venation it may be assumed that the same is true for the hemizygotes of $R^5(+)$, $R^8(+)$, and $R^{21}(+)$, which in heterozygotes proved to be more closely normal than the former named alleles. For the same reason and in agreement with the normal phenotype of $R^{15}(+)/R^{15}(+)$, and of the two other $R(+)$ homozygotes studied by DUBININ and SIDOROV, it may be assumed that the homozygotes of $R^5(+)$, $R^8(+)$ and $R^{21}(+)$, if viable, will cause normal phenotypes. The deficient venations caused by the heterozygous constitutions $R^5(+)/+^3$, $R^8(+)/+^3$, and $R^{21}(+)/+^3$ may therefore be regarded as further

cases of negative heterosis, by comparison with both the homo- and hemizygotes, due to heterozygosity at the *ci* locus.

It is clear that there is a decrease in effectiveness of *both* the $+^3$ and the $R(+)$ alleles since the heterozygotes are more deficient in venation than either $+^3$ or $R(+)$ hemizygotes. Such decrease in phenotypic effectiveness is in contrast to the observations that increases in gene dosage from one mutant *ci* to three *ci* alleles lead to increasingly less deficient phenotypes, as do increases in dosage (at 14°C) from one $+^C$ or one $+^3$ to two $+^C$ or two $+^3$ alleles (STERN 1943). The increasingly more closely normal venation covered in these dosage experiments extends over the same range in which the decrease in effectiveness of heterozygous combinations occurs, as reported in the present paper.

The mechanism of this decrease of effectiveness is a matter of speculation. Conceivably, a kinetic scheme may be invented according to which some or each of the different alleles exert both vein promoting and inhibiting influences, and where these opposing influences operate at different levels of saturation curves. On the other hand the decrease of allelic effectiveness in certain combinations may be the result of a mutual antagonism of the alleles involved. If this is the case, it may be inferred from a consideration of $R^{15}(+)/+^3$, that the mutual inhibition is probably not due to direct interaction of the chromosomal alleles themselves. $R^{15}(+)$ represents an insertion of an excised section of chromosome 4 into the middle of the left arm of chromosome 2 (STERN, MACKNIGHT, and KODANI 1946). The insertion carries the $R^{15}(+)$ allele and, in salivary gland nuclei, does not pair with the homologous region of the intact chromosome 4. It may be assumed that no pairing occurs in the nuclei of other cells either so that there is not sufficient proximity between the loci of $R^{15}(+)$ and of $+^3$ to permit a direct interaction. The indirect interaction may be thought of in terms of competition at later developmental stages between different products of the action of the different alleles. As an alternative it may consist of a mutual, partial inactivation of the alleles by their products or in a mutual antagonism of the products themselves which might result in their partial inactivation. Whatever the interpretation, it must finally include the fact that a decrease in effectiveness may occur in the transition from hemizygous to homozygous constitution: hemizygous $R^2(+)$ veins are normal while homozygous $R^2(+)/R^2(+)$ are deficient (STERN, MACKNIGHT, and KODANI 1946). Moreover it should be noted that a decrease in allelic effectiveness is not restricted to genotypes containing position alleles. A case similar to the interaction of $R(+)$ alleles and $+^3$ has been described earlier for the spontaneous mutant allele ci^W, and $+^3$: hemizygotes for ci^W and $+^3$ are nearly or fully normal, but the heterozygotes $ci^W/+^3$ are greatly deficient in venation (STERN and SCHAEFFER 1943a). Similar to the case of $R^2(+)$, homozygous ci^W flies are more deficient in venation than are hemizygous ci^W individuals.

As unusual as the phenomenon described in this report may appear it does not stand completely alone. The heterosis at the *ci* locus of Drosophila may be compared with the situation in Habrobracon (WHITING 1943) where hemi- and homozygotes for different sex alleles result in maleness, while heterozygotes for these alleles develop into females. In the guinea pig the coat color

caused by certain genotypes is more intense brown in *Pp* than in either *PP* or *pp* animals (WRIGHT 1947). It may be pointed out further that mycelia of *Neurospora crassa* heterocaryotic for the sex alleles *A* and *a* show a different growth from that of homocaryotic *A* and *a* cultures (BEADLE and COONRADT 1944). Finally, the interaction of the *ci* alleles offers similarities with that of sterility alleles in *Oenothera organensis* (D. LEWIS 1947) where two alleles in heterozygous diploid pollen permit tube growth in styles on which haploid pollen containing either of the constituent alleles does not grow.

SUMMARY

Hemizygotes and at least one type of homozygotes for a series of position alleles $R(+)$, at the cubitus interruptus locus of *Drosophila melanogaster*, possess normal venation, as do hemizygotes and homozygotes for the normal allele $+^s$ of this locus. Heterozygotes $R(+)/+^s$ exhibit various degrees of deficient venation. Thus decreases in effectiveness of the alleles $R(+)$ and $+^s$ occur when they are joined together in one genotype. These decreases furnish cases of negative heterosis due to heterozygosity at a single locus. It is pointed out that a distinction needs to be made between heterosis by comparison with the homozygotes and by comparison with the hemizygotes.

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